THE EFFECT OF PRE-PUBERTAL EXPOSURE TO DI-(N-BUTYL) PHTHALATE (DBP) ON CELL PROLIFERATION IN ADULT MALE JAPANESE QUAIL (*Coturnix coturnix japonica*) BRAINS

By:

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DECLARATION

I ……., hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise). It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. I declare that neither the whole work, nor any part of it, has been submitted for another degree or examination at this or any other university.

Signature: ……

Date: …….

DEDICATION

‘You are not even a doctor you are just a mom!’

My work is dedicated to my wonderful children, Wanda, Una, Nono, Kusa, my true fans and my worst critics; for their encouragement to keep going and their criticism when I stop giving them attention because I think I am a doctor and professor when I am just a mum.

To God Almighty, for giving me the wisdom, the clarity, the strength and the spirit to persevere in juggling both parenting and studying.

ABSTRACT

Neurogenesis is a process that comprises neuronal progenitor cell proliferation, migration differentiation, and integration of young neurons into existing neuronal circuits. Neurogenesis has been observed in avian species, including quail. Neuronal cell proliferation, which is part of adult neurogenesis, was investigated using cellular markers DCX and PCNA, in the hippocampus, mesopallium, nidopallium, medial striatum, medial pre-optic nucleus and ventricles of 14 week old male Japanese quail brains. This was after a 30 day pre-pubertal exposure to the environmental contaminant and endocrine disruptor di(n-butyl) phthalate (DBP), a well-known neuronal development disruptor and neuronal toxicant. Thirty birds were randomly grouped into groups of four (n=4 each) and were intragastrically fed DBP dissolved in corn-oil. The control was fed corn oil only, while three groups were fed 10 mg/bodyweight, 50 mg/bodyweight, 400 mg/bodyweight, effectively dividing them into a low medium and high dose groups. The birds were euthanised using carbon dioxide, brains were harvested and post fixed in a 1:1 mix of ethylene glycol and glycerol (antifreeze) in 0.244M PB (phosphate buffer) and stored at -20 ^{0}C. Repeated five series coronal sections of 50 µm were cut in a rostro-caudal direction using a freezing microtome. The 5th series sections were stained with cresyl violet for analysis of brain cytoarchitecture, while 3rd and 4th series sections were stained with DCX and PCNA. Intense staining was observed along the ventricles for both PCNA and DCX, and some PCNA-ir cells were present in the POM. DCX-ir cells were observed in the pallial brain areas of both treated and control groups. Statistically significant differences (p= 0.0001) in counts of DCX-ir between the control and treated groups in the five brain regions were observed, but there were no statistically significant differences (p=0.886) between the brain regions themselves across all dosage groups. DBP treatment affected DCX-ir cell counts in all brain areas under study, irrespective of brain area or dosage. There were significant differences (p=0.0067) of PCNA-ir cell counts between the treated groups and the control for the POM (p=0.0067) and PCNA-ir cell counts between the control and ventricles (p=0.0001). DBP affected cell proliferation in the ventricles and the POM.

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ac	Anterior Commissure
ADHD	Attention-Deficit/Hyperactivity Disorder
AEC	Animals Ethics Committee
ARO	Aromatase
BDNF	Brain Derived Neurotrophic Factor
BSA	Bovine serum albumin
BSTL	Lateral bed nucleus of the stria terminalis
CAS	Chemical Abstracts Service
DAB	3,3'-Diaminobenzidine
DBP	Di(n-butyl) phthalate
DCX	Doublecortin
DCX-ir	Doublecortin immuno-reactive cells
DDT	dichloro- diphenyl-trichloroethane
DEHP	Di(2-ethylhexyl) phthalate
DMRB	Digital Media Ring Buffer
DNA	Deoxyribonucleic acid
E	Entopallium
EDCs	Endocrine-Disrupting Chemicals
EPA	Environmental Protection Agency
EPI	Endogenous peroxidase inhibitor
ERα	Oestrogen receptor alpha
ERβ	Oestrogen receptor beta
FPL	Lateral fore-brain bundle
GP	Globus pallidus

HA	Hyperpallium Apicale
Hp	Hippocampus
HPA	Hypothalamus-pituitary-adrenal axis
HPG	Hypothalamus-pituitary-gonad axis
HPT	Hypothalamus-pituitary-thyroid axis
ILO	International Labour Organisation
IMM	Intermediate Medial Mesopallium
IPCS	International Programme on Chemical Safety
LaM	Lamina mesopallialis
LFS	Lamina frontalis superior
LPS	Lamina pallio-subpallialis
LSt	Lateral striatum
M	Mesopallium
MAP	Microtubule associated phosphoprotein
MAP2	Microtubule associated protein
MBP	Mono butyl phthalate
MEHP	Mono(2-ethylhexyl) phthalate
MSt	Medial striatum
N	Nidopallium
NEED	Neuroendocrine Effects of Endocrine Disruptors
OECD	Organisation for Economic Cooperation and Development
PB	Phosphate buffer
PCNA	Proliferating cell nuclear antigen
p-CREB	Phosphorylated c-AMP responsive element- binding protein
PEs	Phthalate esters
POM	Medial preoptic nucleus;
PVC	Polyvinyl chloride

RNA	Ribonucleic acid
ROS	Reactive oxygen species
SANS	South African National Standards
TSM	Tractus septopalio mesencephalicus
US ATSDR	United States Agency for Toxic Substances and Disease Registry
UNEP	United Nations Environment Programme
VL	Lateral ventricle
VZ	Ventricular zone
WHO	World Health Organisation

1 CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

In recent years, there has been growing scientific, media and public concern over health threats caused by synthetic chemical pollutants that are pervasive and persistent in our environment. These chemical pollutants or their synthetic analogues, otherwise known as Endocrine-disrupting chemicals (EDCs), have the potential to alter or interfere with normal endocrine function, which is directly linked to hormone biosynthesis, transportation, receptor function, and with the overall effects on physiology, behaviour, development and reproduction in both wildlife and human populations (Colborn et al., 1998; DeRosa et al., 1998; US ATSDR, 2001; Bergman et al., 2012).

Humans and wildlife populations are continuously exposed to EDCs or a cocktail of these compounds, which are constantly discharged into the atmosphere and natural aquatic bodies, including soil, air, households, work and even school environments (O'Shea et al., 1980; Kavlock et al., 2002) where exposure is typically via inhalation, ingestion, and dermal contact (Heudorf et al., 2007).

Studies in mammals and lower vertebrates have suggested that EDCs can cause disorders of neuronal differentiation, as well as disrupt the development and function of neurological organs (Li et al., 2013; Li et al., 2014). Therefore, it is due to safety related issues and arbitrary use of these synthetic chemicals that concerns have been raised by the International Programme on Chemical Safety (IPCS), and these concerns stemmed from the following factors; unexplained increases in endocrine related diseases (where genetic factors had been ruled out); declines in wildlife

populations exposed to these chemicals; a data base of close to 800 known or suspected EDCs which remain untested; non-validated laboratory test methods to identify specific EDCs and their effects; the susceptibility of organisms to EDCs during foetal development and puberty, the effects of EDCs on brain development which is linked to neuropsychiatric disorders such as autism, attention-deficit/hyperactivity disorder (ADHD), and learning disabilities.

The IPCS, established in 1980, is a joint venture of the United Nations Environment Programme (UNEP), and the International Labour Organisation (ILO) and the World Health Organisation (WHO) where the overall objective is to establish the scientific basis for assessment of risk to human health and the environment from exposure to chemical pollutants. It also recognises that this knowledge gap compromises the protection of both humans and wildlife species from the harmful effects of EDCs (Damstra et al., 2002). Indeed, EDCs are now widely recognised as a major environmental issue. Urbanised and industrialised areas have high concentrations of these chemical pollutants (Hodge and Diamond, 2009). The constant production of EDCs, excessive usage, bioaccumulation in water, soil, air and living organisms, coupled with the ability to spread via oceans, air currents and international exchange of goods, leads to global exposures (Bergman et al.,2012; Kajta and Wojtowicz, 2013).

Studies on EDCs as isolated chemicals and/or combinations and their mechanistic effects on humans and wildlife populations are still a grey area, where links between specific EDCs/combinations need to be linked to specific species and tissues. Interestingly, the indiscriminate and significant use of pesticides such as dichloro-diphenyl-trichloro-ethane (DDT) and [2,2-bis-(p-chlorophenyl)-1,1-dichloroethylene] (DDE) in the 1950s was correlated with egg shell thinning in birds and is probably

the best example of reproductive impairment that caused a severe population decline in a number of raptor species in Europe and North America (Koeman et al., 1972; Peakall et al., 1973; Odsjo and Sonnel, 1982; Vos et al., 2000), and which was found to be induced by embryonic exposure to EDCs (Axelsson, 2008). Importantly, studies on the effects of EDCs on brain tissue, particularly in avian species, are still lacking. The chemical DBP (di(n-butyl) phthalate) under study, a phthalate ester, classified as an EDC, is implicated as a potential neurotoxic agent (Li et al., 2014). It is suggested that exposures tend to be greater during foetal development, the perinatal period, puberty and childhood, hence the need to understand exposures during specific developmental periods like the pre-pubertal period (Damstra et al., 2002; Diamanti-Kandarakis et al., 2009; Bergman et al., 2013). Therefore, endocrine related effects may be attributed to specific species, developmental stages, specific tissues, specific chemicals and dosage paradigms.

1.2 Background

The field of endocrine disruption has been controversial with both sceptics and endocrinologists being equally vocal concerning the harmful effects of EDCs, especially in determining which chemicals are toxic at what doses, which developmental stages are affected, what tissues are affected, what is the period of latency and what individual variations are experienced (Damstra et al., 2002; Diamanti-Kandarakis et al., 2009; Bergman et al., 2013). The controversy is further compounded by difficulties in extrapolating data derived from in vitro studies and wildlife cases to humans and a lack of sensitive test methods and test guidelines (Beronius et al., 2009; Gore and Patisaul, 2010). Before the terminology of EDCs was introduced, neuroendocrinologists had long developed the concept that

interference with endogenous hormones during critical developmental periods leads to permanent behavioural and physiologic changes in adulthood, whereby disruption of pathways regulated by hypothalamic neuro-endocrine circuits disrupts the homeostasis of neuroendocrine processes, therefore indirectly predicting the neuroendocrine disrupting effect of EDCs (Gore and Patisaul, 2010; Bergman et al., 2012). Central neuroendocrine systems are targets for EDCs (Gore, 2010).

1.3 What are Endocrine Disrupting Chemicals?

The term endocrine-disrupting chemicals or EDCs was first introduced in 1991 at the Wingspread conference centre in Racine, Wisconsin, USA. According to the U.S Environmental Protection Agency (EPA, 2006), endocrine-disruptors are defined as *'chemicals that either mimic or block the effects of hormones at the target receptor/tissue or by directly stimulating or inhibiting production of hormones by the endocrine system'*, whereas Bergman et al (2012) defines EDCs as '*exogenous substances or their mixtures that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny or (sub) populations'*. These exogenous substances interfere with normal functioning of the endocrine systems of both humans and wildlife; and by mimicking the action of endogenous hormones, antagonise the effects of hormones, stimulate or inhibit production, transportation, or degradation of hormones which consequently result in altering hormonal homeostasis, causing an imbalance or disturbance in the regulation of development, growth, reproduction and behaviour (Jensen et al., 1995; Crisp et al., 1998; Jobling and Tyler, 2006; Verma and Rana, 2009).

According to Waye and Trudeau (2011), EDCs should be viewed in a broad context that is not only confined to hormonal systems but viewed as environmental pollutants that can impact brain function in relation to hormonal systems; hence they propose

the introduction of the term ‘neuroendocrine disruption’ and the introduction of the concept of ‘Neuroendocrine Effects of Endocrine Disruptors’ (NEED). Therefore, neuroendocrine disruptors can be viewed as pollutants in the environment that are capable of acting as agonists/antagonists or modulators of the synthesis and/or metabolism of neuropeptides, neurotransmitters, or neurohormones which subsequently alter diverse physiological, behavioural or hormonal processes eventually affecting an animal’s capacity to reproduce, develop, grow and deal with stress and other challenges in its environment (Waye and Tradeau, 2011).

Central neuroendocrine systems control homeostatic processes like growth, reproduction, metabolism, lactation, stress response, and energy balance, where signals are initiated in the hypothalamus and conveyed initially to neural effectors then endocrine effectors that link the brain with peripheral endocrine systems. Neuroendocrine systems integrate hormone based endogenous signals which control processes such as feedback mechanisms, metabolic pathways, pheromones, temperature, photoperiods, population stresses which, in turn, result in behavioural, physiological and environmental adaptation of all organisms. Therefore, neuroendocrine disruption transcends basic hormonal disruption to affect other neurochemical pathways responsible for physiological and behavioural processes where the end result is an altered endocrine phenotype (Gore, 2010; Gore and Patisaul, 2010; Waye and Tradeau, 2011)

Exposures to EDCs are implicated in a variety of neurological disorders such as autism, ADHD, and learning disabilities, whereby there is alteration of neural transmission and formation of neural networks (Kajta and Wojtowicz, 2013). Therefore, exposure to EDCs during early life can disrupt normal patterns of

development and thus alter brain function and disease susceptibility later in life (Schug et al., 2015).

Public concerns around the effects of EDCs on brain function, and neuropsychiatric disorders such as autism, attention deficit hyperactivity disorder (ADHD), and learning abilities, appears to be mounting. According to Waye and Trudeau (2011), chemical pollutants can affect an organism at brain, pituitary, endocrine and/or target tissue level, interfering with the hypothalamus-pituitary-gonad (HPG) axis, hypothalamus-pituitary-thyroid (HPT) axis, hypothalamus-pituitary-adrenal (HPA) axis, resulting in physiological and behavioural changes.

There is reciprocal regulation between the various pathways (Dobson et al 2003; Hogan et al., 2007); therefore, a chemical pollutant that mimics or disrupts one specific neuroendocrine pathway will affect the other (Waye and Trudeau, 2011).

1.3.1 Sources and routes of exposure to EDCs

Manufacturers worldwide do not declare chemical constituents of their products, materials and goods, except for pharmaceuticals, making the identification of products containing chemicals with endocrine disrupting properties a challenge (Bergman et al., 2012). EDCs with a long half-life are persistent in the environment, they bio-accumulate in humans and wildlife to achieve high concentrations in organs and tissues while other less persistent EDCs with a short half-life are non-bio-accumulative but are still problematic because the exposure is continuous (Volkel et al., 2002).

EDCs can affect the endocrine system in their original form or metabolised into active metabolites. Molecules with endocrine disrupting properties are very diverse, they include synthetic chemicals like industrial solvents, lubricants, plastics,

plasticisers, pesticides, pharmaceutical agents and natural chemicals like phytoestrogens found in animal and human foods like infant soya milk (Damstra et al., 2002; Diamanti-Kandarakis et al., 2009; Gore and Patisaul, 2010; Bergman et al., 2012). Cosmetics and personal care products like shampoos, lotions, nail varnish, hair products, toothpaste, soaps, and lotions contain solvents, fragrances, plasticisers, preservatives, antimicrobials, stabilisers and metals as additives that have endocrine-disrupting properties. In addition, products found in households, workplaces, and schools like cleaning agents, electronics, furniture, carpets, building materials, paints, paper, clothing, toys, garden chemicals and herbicides also contain additives (preservatives, solvents, dyes, stabilisers, antimicrobials) with endocrine disrupting properties (Damstra et al., 2002; Sathynarayana et al., 2008; Bergman et al 2012).

Human exposure to known EDCs, potential EDCs and their precursors is via inhalation, ingestion (food and water) and dermal contact (cosmetics, floors, clothing). Children are particularly vulnerable because of their floor play, resulting in inhalation of dust particles in their home and school environment and hand-to-mouth exploration of objects and toys. Exposure can also be in-utero via the placenta as well as breast milk (Damstra et al.,2002; Sathynarayana et al.,2008; Bergman et al., 2012). Wildlife exposure comes from contaminated air, water, food, soil, depending on the half-life of each contaminant where persistent EDCs can be transported by natural processes (oceans and air currents) to affect wildlife in remote non-industrialized ecosystems (Damstra et al., 2002; Bergman et al., 2012).

EDCs reach our environment during manufacturing, usage and disposal of materials. During manufacturing, chemicals are added to materials and emissions are released

into air, water and soil. Emissions are also released during incinerations at dump sites and recycling of electronics (Bergman et al., 2012; Heackock et al., 2016).

House-hold products used for cleaning, bathing, and medicinal purposes enter the sewage system to enter aquatic environments, contaminating water and soil. Runoff from agricultural fields contaminated with pesticides, fungicides, hormones, and pharmaceuticals is another source of EDCs. Bio-solids harvested from recycled sewerage used as fertilisers contain EDCs (Bergman et al., 2012).

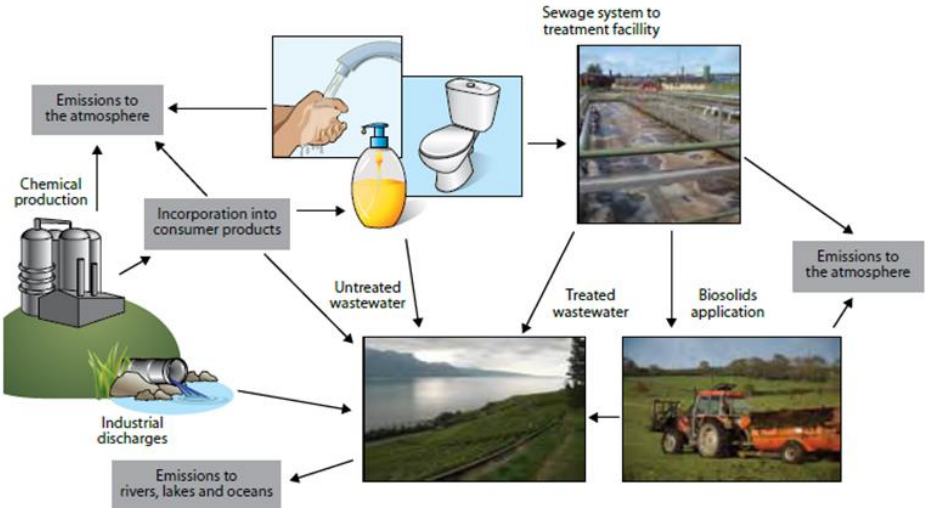


Figure 1.1: Diagrammatic representation of how humans and wildlife are exposed to EDCs (adapted from Bergman et al., 2013). This image is copied in terms of "fairdealing" in terms of section 12 of South African copyright act of 1978 (as amended).

Pharmaceuticals and their metabolites consumed by humans can be detected in surface water and effluents, which include hormones, antidepressants, anti-inflammatories, beta blockers, anti-cancer medicines, anti-epileptics and lipid regulators (Monteiro and Boxall, 2010).

1.3.2 How EDCs interfere with hormones and their physiologic effects

The endocrine system comprises a series of ductless glands (pituitary, thyroid, parathyroid, pineal, suprarenal, gastrointestinal, gonads, adipose tissue, heart muscles) that secrete hormones directly into the bloodstream.

A hormone is defined as ‘a molecule produced by an endocrine gland and transported via the bloodstream to cause its effect on distant cells and tissues’ (Melmed et al., 2011).

Peripheral endocrine organs release hormones directly into the bloodstream to regulate homeostasis in a negative feedback mechanism, which, together with hypothalamic neuroendocrine cells, control several major physiologic processes like embryonic cell differentiation, organ formation, adult tissue formation and organ function. Both hormones and neuroendocrine cells act on the brain to coordinate physiologic processes and associated behaviours (Gore, 2010; Melmed et al., 2011).

EDCs will affect all physiological systems and endocrine systems. For example, weight gain is a complex physiologic system that is influenced by endocrine disruptors (Casals-Casas and Desvergne, 2011; Bergman et al., 2012). These chemicals can disrupt hormonal effects by interfering with proteins responsible for production/synthesis, transportation, timed delivery of hormones to specific organs and tissues or act on hormone receptor complexes to alter physiologic effects on organisms (Bergman et al., 2012)

EDCs act on nuclear hormone receptors, (oestrogen, androgen, progesterone, thyroid, retinoid) non-nuclear hormone receptors, (membrane oestrogen) non-steroid hormone receptors, (serotonin, dopamine, non-epinephrine) orphan receptors, (aryl hydrocarbon) enzymatic pathways responsible for biosynthesis and metabolism of

steroid hormones, and other endocrine and reproductive pathways. Some classes of EDCs have a phenolic moiety that mimics steroid hormones facilitating the binding to steroid hormone receptors where they can act as agonists or antagonists resulting in androgenic, anti-androgenic, thyroid hormone agonists/antagonists, oestrogenic or anti-oestrogenic effects (Takeuchi et al., 2005; Diamanti-Kandarakis et al., 2009; Li et al 2013; Chen et al., 2014). Endogenous hormone receptors have a high affinity for their natural ligand and a lower affinity for endocrine-disruptors.

However, EDCs can still have high potencies at low affinities (Ruenitz et al., 1996). Some endocrine disruptors have similar or greater affinities that can surpass the natural ligands (Grun and Blumberg, 2006).

All hormone sensitive physiological systems are susceptible to EDCs where EDCs are implicated in neurologic disorders (ADHD, autism, learning disabilities, depression), reproductive abnormalities (oligospermia, testicular tubular atrophy, testicular cancer, early breast development, precocious puberty, breast cancer), interference with thyroid metabolism, altered growth patterns, obesity, diabetes, asthma and imbalanced sex ratios (Diamanti-Kandarakis et al., 2009; Matsunaga et al., 2010; Bergman et al., 2012; Bello et al., 2014).

1.3.3 Understanding the key mechanisms of endocrine disruption

Clinicians and researchers need to be alert and sensitive to a population's disease pattern which may be due to environmental factors. Because endocrine disruption represents a complex form of toxicity, researchers need to be careful during interpretation and quantification of results when assessing risk to humans and wildlife. Some cells, tissues and developmental time points are more sensitive to EDCs (Diamanti-Kandarakis et al., 2009).

The age of exposure is critical in endocrine disruption since effects of developmental exposure persist throughout life due to their effects on cell programming, cell differentiation, tissue development, where previously exposed tissues are more susceptible to disease in adult life hence the introduction of the terminology "developmental basis of adult disease" (Diamanti-Kandarakis et al., 2009).

This concept implies there is a period of latency between exposure early in life and manifestation of disease in adult life and aging. Therefore, a developing organism's environment (maternal, egg, external) will interact with its genes to determine an organism's susceptibility to develop a disease later in life (Diamanti-Kandarakis et al., 2009; Bergman et al., 2012). Effects of EDCs are not confined to exposed organisms, but affect offspring and their progeny through epigenetics causing trans-generational epigenetic effects that are transmitted by modifying factors that regulate gene expression, as well as causing mutations of the DNA sequence (Anway et al., 2005).

EDCs are known to have a 'cocktail effect' where EDCs from different classes may have an additive or synergistic effect to elicit the so called 'something for nothing' principle where individual low potency chemicals do not cause harmful effects until combined with other chemicals. This cocktail effect poses a challenge for regulatory bodies that focus on single chemical experimental doses (Damstra et al., 2002; Bergman et al., 2012).

Natural hormones exhibit non-linear and non-monotonic dose response curves because they act on receptor sites which become saturated, and they act at low doses because of their high affinity for their receptors. EDCs exhibit similar properties, however they act at low doses at low affinities for natural hormone

receptor sites and exhibit non-linear and non-monotonic dose response curves which are influenced by receptor abundance, and different affinities for different receptor isoforms (ERα or ERβ) which explains why tissues, cells, and developmental stages manifest different sensitivities to different types of EDCs (Bergman et al., 2012; Vandenberg et al., 2012).

1.4 Phthalate-esters (Phthalates) - as a class of EDCs

Phthalate esters (PEs) are chemical pollutants with endocrine disrupting properties (Latini, 2005). PEs are synthetic chemicals used primarily as plasticiser additives in many industrial compounds, children's toys, personal care products and biomedical devices (Hauser et al., 2004; Latini, 2005; Sathyanarayana et al., 2008; Oehlmann et al., 2009; Rael et al., 2009), to improve flexibility to polyvinyl chloride (PVC) (US ATSDR, 2001). Phthalates are not covalently bound to the plastic polymers, and therefore are continuously released into the air or leach into liquids, which lead to exposure through ingestion, dermal exposure and inhalation, reaching both humans and wildlife (Heudorf et al., 2007; Sathynarayana et al., 2008; Holahan and Smith, 2015).

Following oral ingestion, phthalates are rapidly hydrolysed in the gastro-intestinal tract and other tissues by non-specific esterases to produce the corresponding monoesters and alcohol (Thomas and Thomas, 1984; Mentlein and Butte, 1989), which enter systemic circulation through portal blood (EPA, 2006). Molecular studies have shown PEs do not only undergo hydrolysis but redox reactions, resulting in electron transfers to generate reactive oxygen species (ROS) leading to oxidative stress in living organisms which results in organ damage, carcinogenesis, inflammation, immunosuppression and interference with cell signalling (Kovacic, 2010).

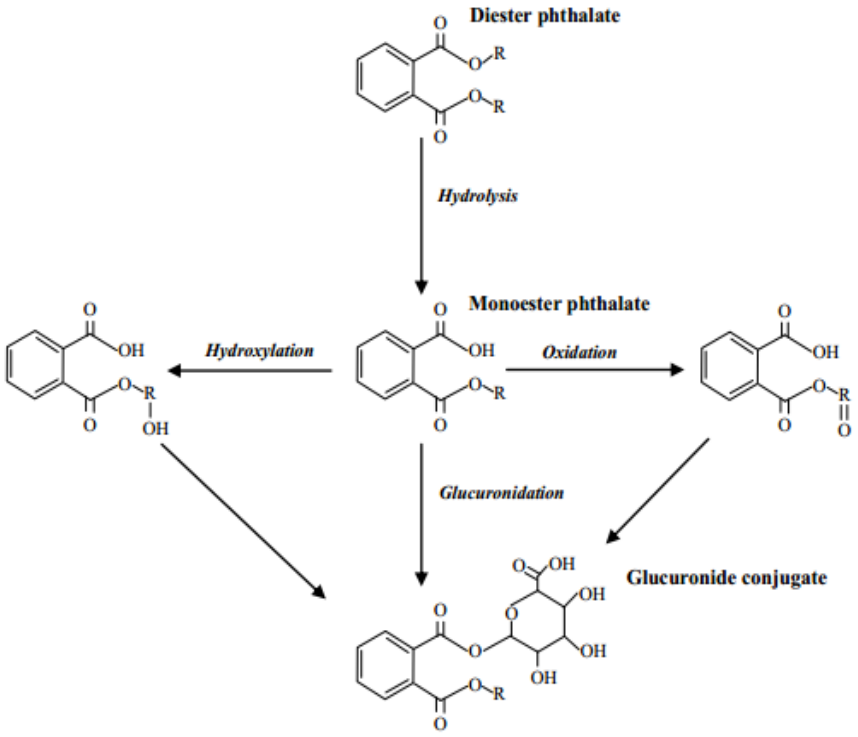


Figure 1.2: Schematic overview of the general metabolic pathway for phthalates (modified from (Frederiksen et al., 2007). This image is copied in terms of "fairdealing" in terms of section 12 of South African copyright act of 1978 (as amended).

Epidemiological evidence also suggests that phthalates can cross the placental barrier and are expressed in breast milk, resulting in perinatal and *in-utero* exposure (Shea, 2003; Thundiyil et al., 2007).

Children are particularly vulnerable due to their exploratory floor play, hand to mouth behaviour and sensitive developmental periods of both nervous and reproductive systems. Infant exposure to lotion, powder and shampoo was associated with high urine phthalate metabolites (Sathyanarayana et al., 2008). They have also been found to cause seminiferous tubule atrophy and premature breast development in girls (Diamanti-Kandarakis et al., 2009).

Thus, phthalate plasticisers are one of the most abundant man-made pollutants with wide spread attention from the scientific community, regulatory bodies and public

media, where there has been a great debate regarding their safety to humans (Kovacic, 2010).

DBP, a phthalate ester is a ubiquitous environmental contaminant and enhanced-oestrogenic endocrine distruptor that affects reproduction, development, neuronal development and is potentially carcinogenic (Matsunaga et al., 2010; Chen et al., 2014; Li et al.,2014). DBP is metabolised into the monoester, mono-butyl-phthalate (MBP), which is considered to be a potential neurological toxicant (Li et al., 2013). The mono ester, mono-butyl-phthalate (MBP) undergoes further glucuronidation in the liver, where both free and glucuronidated MBP circulate in serum, and is subsequently excreted in urine. The general metabolic pathway of phthalates is shown in Figure 1.2 above.

1.5 EDCs and phthalates affect neurogenesis

Adult neurogenesis is comprised of birth, migration and integration of young neurons into existing mature neuronal circuits to improve brain function (Patzke et al 2013). Oestrogen has been shown to promote neural progenitor cell proliferation both *in vitro* and *in vivo* in the rat hippocampus and its neurotropic effects influence neuronal differentiation and migration in the avian brain (Lee et al., 2007; Handa et al., 2012). DBP has oestrogenic effects that affect neuronal development (Li et al., 2014). EDCs like DBP interact with hormone/receptor complexes to alter developmental processes and steroid hormones (oestrogen and testosterone), that modulate neurogenesis, which involves development, differentiation, synaptic plasticity, neuronal excitability, neuronal survival, and axonal outgrowth via various mechanisms that include binding to ERα and ERβ found in brain tissue (Colborn et al., 1998; Morissette et al., 2008; Frye et al., 2012). EDCs cause a disturbance to dendritic growth on hippocampal neurons in early development which may lead to

psychological disorders; they bind to microtubule associated protein-2(MAP2), which is localised in dendritic neurons where MAP2 promotes dendritic assembly and stability. MAP2 is a high affinity target for EDCs (Matsunaga et al., 2010).

1.5.1 DBP induces neurotoxicity

DBP, a recognised environmental oestrogen, was found to have enhanced-oestrogenic activity *in vivo* and had both oestrogenic and anti-androgenic activity *in vitro* (Chen et al., 2014; Li et al., 2014). In brain tissue, endogenous oestrogen binds to ERα and ERβ to exert its neuroprotective effects of promoting neurogenesis and synaptic plasticity to promote memory and cognitive behaviour (Qu et al., 2013). In an oestrogenic endocrine disrupting activity test on zebra fish embryo, DBP was found to be highly toxic and demonstrated an enhanced oestrogenic activity (Chen et al., 2014).

DBP exposure was shown to induce cell apoptosis, reduced expression of synaptophysin, reduced post synaptic excitability of hippocampal neurons in neonatal and immature rat offspring following perinatal exposure, but had no effect on mature offspring (Li et al., 2013). The active metabolite mono-(2-ethylhexyl) phthalate or MEHP, a sister compound of di-(2-ethylhexyl) phthalate or DEHP was found to promote neurocyte differentiation, but suppressed neurocyte proliferation *in vitro* (Chen et al., 2011).

Molecular studies have shown DBP to up-regulate the expression of ARO (aromatase converts androgens to oestrogens) and down regulate the expression of ERβ, BDNF (brain derived neurotropic factor) and p-CREB (phosphorylated c-AMP-responsive element binding protein). ERβ, BDNF, p-CREB modulate brain

development, synaptic plasticity and improve memory (Li et al., 2014). Isoflavone phyto-oestrogens also recognised as environmental oestrogens and endocrine disruptors were found to be anti-oestrogenic, act on ERβ and ERα dependant gene expression in the brain to suppress oestrogen mediated neuro-behaviour (Patisaul et al., 2010).

Therefore, in this study, we postulate that DBP will affect endogenous steroid hormone homeostasis and induce its neurotoxic effect by altering neuronal cell proliferation and differentiation in various telencephalic brain areas of the adult male Japanese quail exposed during the pre-pubertal period of sexual differentiation and development.

1.6 Avian adult neurogenesis and neurogenic sites

Neurogenesis (progenitor cell proliferation, migration, differentiation, integration, apoptosis) was initially thought to be restricted to embryonic development in mammals, however recent studies confirm it occurs within the ventricular zone (VZ) of adult vertebrate brains like birds, mammals and humans where mammalian brains experience a gain in neuron numbers while in avian species older neurons are replaced by younger neurons (Barnea, 2009). Seasonal variations of song learning, spatial learning (associated with food storage), migratory behaviour, and socialisation are all associated with memory dependant behaviour in avian species which appear to be regulated by adult neurogenesis (Barnea, 2009).

Songbird species like the canary (*Serinus canaria*) and zebra finch (*Poephila guttara*) have been used extensively to study adult neurogenesis (Brenowitz, 2008; Nottebohm, 2008). These studies focused on neuronal proliferation along the VZ, neuronal migration and integration into telencephalic circuits responsible for the control of song production. Other studies by Alvarez-Buylla (1992) on canaries

(*Serinus canaria*) also confirm the VZ as a neuronal birth site, where many regions of the adult encephalon receive new neurons originating from the VZ. Avian neurogenic hotspots are found in the lateral VZ bordering the hippocampus in a rostro-caudal direction (Patel et al., 1997; Barnea et al., 2011). These hotspots give rise to stem cell progenitors that undergo mitosis where one daughter cell differentiates into a young neuron that migrates to specific brain areas and integrates into existing neuronal circuits. Young neurons separate from the VZ and elongate to penetrate the adult brain parenchyma at a high rate of 26 µm/hr (Alvarez-Buylla, 1992; Alvarez-Buylla et al., 1994).

It is now recognised that extensive neurogenesis is not only confined to songbirds but also found in other avian species and orders like the quail (*Coturnix japonica*) (Nikolakopolou et al., 2006; Balthazart et al., 2010), ring doves (*Streptopelia risoria*) (Ling et al., 1997; Cao et al., 2002), rock pigeons (*Columba livia*) (Melleu et al., 2013). Neurogenesis unrelated to spatial memory or vocal control, was also observed in adult chickens (*Gallus gallus*), ringdoves (*Streptoplia risoria*) and rock pigeons (*Columba livia*), possibly associated with defensive behaviour and filial imprinting (Nikolopoulou et al., 2006). Quails belong to the order Galliformes and genus Phasianidae; they are neither song birds nor food storing birds (Balthazart and Ball, 2014), however they exhibit spatial orientation and memory to recall specific sites (Ruplo et al., 2011).

Quail exhibit crowing, a non-learned but testosterone sensitive sexually differentiated behaviour as opposed to learned song production (Nikolakopoulou et al., 2006).

Adult neurogenesis contributes to the plasticity of the hippocampal circuitry to augment memory (Patzke et al., 2013). The avian hippocampus is crucial for spatial

memory and memory based retrieval of food caches (Zeigler et al., 1993). According to Atoji and Wild (2006), the avian hippocampus is the pallial area medial to the paraventricular sulcus. Studies on sparrows (*Zonotrichia leucophys*) showed a higher hippocampal neurogenesis on migratory than non-migratory birds while studies on food storing chickadees (*Poecille atricapillus*) showed a higher hippocampus neurogenesis during autumn and winter, suggestive of a need for enhanced memory to recall food caches (Melleu et al., 2016). In songbirds, specific parts of the telencephalon, particularly the nidopallium, are known to incorporate new neurons in adulthood, where two thirds of these new neurons are fusiform unipolar or fusiform bipolar. The fusiform neurons are young migrating neurons, while the rest are round multipolar neurons, which are older neurons at an early stage of differentiation (Boseret et al., 2007; Balthazart and Ball, 2014). The hippocampus is a neurogenic hotspot in pigeons (*Columba livia*) and is responsible for homing, averse conditioning, cognitive behaviour and spatial memory (Barnea et al., 2011; Melleu et al., 2013). Non-oscine birds display numerous immature neurons in the prosencephalon unrelated to spatial learning, memory or vocal control, especially within the IMM (intermediate mesial mesopallium), where the IMM is responsible for learning, formation and retention of memory in non-oscine birds (Nikolakopolou et al., 2006; Melleu et al., 2013).

The avian pallium occupies 75% of the telencephalon, and integrates information similarly to mammalian sensory and motor cortices (Jarvis et al., 2005).

1.7 DCX and PCNA as markers of neurons and cell proliferation

A study on pigeons (*Columba livia*) identified small and fusiform doublecortin reactive (DCX-ir) cells within the VZ and multipolar DCX-ir within the pallial regions (Melleu et al., 2013). DCX is part of the microtubule associated phosphoprotein

(MAP) expressed by neuronal precursor cells in cell cycle and post mitotic neuronal cells. DCX is responsible for neuronal migration, (radial and tangential) dendritic development and dendritic remodelling in both developing and adult brains (Rao and Shetty, 2004; Plumpe et al., 2006; Melleu et al., 2013; Vellema et al., 2014; Melleu et al., 2016). DCX is localised in the cytoplasm therefore expressed in cell bodies and processes of pre-mitotic and post-mitotic cells (Plumpe et al 2006; Vellema et al., 2014). PCNA or cyclin is an intracellular peptide which is used as a marker for the G1 and S phases of the cell cycle and is associated with DNA replication in the cell nucleus (Kelman, 1997; Kurki et al., 1988).

In conjunction with other markers, Proliferation Cell Nuclear Antigen (PCNA) has been used to study brain development and differences in brain cell cycle rates of zebra finch (*Taeniopygia guttata*), northern bobwhite quail (*Colinus virginianus*) and parakeets (*Melopsittacus undulatus*) (Charvet and Strieder, 2008)

The current study investigated changes in cell proliferation, morphologic neuronal cell differentiation and neuronal cell distribution in specific areas of adult male Japanese quail brains following a 30-day pre-pubertal exposure to the potential anti-androgen, phthalate ester, DBP, using PCNA and DCX as cellular markers.

We compared these changes at various doses of exposure. Previous studies have demonstrated a reduced expression of DCX in the rat hippocampus following prenatal exposure to DBP, especially in middle dose groups (Kim and Jeon, 2015).

1.8 The Japanese quail as an animal model of study

Figure 1.3: Reproductive male Japanese quail with cinnamon brown breast feathers exhibiting strutting behaviour (adapted from Ottinger M, 2001; Huss et al., 2008). This image is copied in terms of "fairdealing" in terms of section 12 of South African copyright act of 1978 (as amended).

Quail belong to the order Galliformes and genus Phasianidae. These migratory galliforms have a life span of 2-3 years in the wild and have been used since the 1950s as research subjects (Ottinger, M; 2001; Huss et al., 2008). The Japanese quail (*Coturnix coturnix japonica*) has a number of advantages for its suitability as an avian model species in avian risk assessment studies (OECD, 2010) and is frequently used as an animal model in most avian toxicity tests (Romijn et al., 1995). The advantage of using quail as research subjects and laboratory animals is because of the following factors: because of their precocious nature, they require minimal parental care; their small size requires minimal maintenance in terms of housing and feeding; their short lifespan enables physiologic and aging studies to be conducted within a short period of time; they have a very high egg production, averaging 250 eggs per annum: they are capable of producing 3-4 generations per annum; quail exhibit specific genetic and phenotype characteristics leading to specific quail lines, which reduce confounding factors of genetic variability (Huss et al., 2008; Moregaonkar, 2011; Jaspers, 2015). In this study, confounding factors

were further minimised because the birds were the same sex and age, and were exposed to the same feeding schedule and environment.

Quail embryos and adult quail are used extensively in studies of vertebrate physiology, genetics, nutrition and environmental toxicity testing as models for disease in humans. Because of their appetite for a variety of seeds, worms and insects they are used in environmental toxicity testing of chemicals in the food chain. They have a comparable physiology to humans and higher order animals, therefore toxicology studies can be extrapolated to humans and higher order animals as compared to rodents (Huss et al., 2008; Moregaonkar et al., 2011). Quail have been widely used to study the effect of EDCs on reproduction and behaviour (Haldin, 2005). Hence they were relevant for the current study.

1.9 Justification for the study

There is a worldwide concern from the public, media, regulatory bodies and scientific community concerning the safety of EDCs on both wildlife and humans, where the chemical under study, DBP, a phthalate ester, is one of these EDCs. There are very few studies which have been done in Africa, Asia, Central and South America on the effects of EDCs for both humans and wildlife (Bergman et al., 2012). Pthalate induced toxicity studies on humans have been predominantly epidemiological, while laboratory studies were based on rodents; little is known about phthalate induced toxicity in avian species (Diamanti-Kandarakis et al., 2009). Animal species tend to be susceptible to pthalates during critical developmental periods like foetal development, perinatal periods, peripubertal periods, and early childhood ,where their effects are species specific, cell line specific and therefore tissue specific, resulting in specific tissue sensitivities (Bergman et al., 2012; Vandenberg et al., 2012; Li et al., 2013), which is why our study focuses on male pre-pubertal

Japanese quail brain tissue. There are over 800 chemicals known to have endocrine disrupting properties that act at very low doses and exhibit non-linear and non-monotonic dose response curves (Diamanti-Kandarakis et al., 2009; Bergman et al., 2012; Vandenberg et al., 2012). Studies on EDCs as individual compounds or in combination with other chemicals that correlate dosage to effect are still lacking, which is why our study specifies a dosage regime of DBP into low, medium and high to find the lowest possible effective dose. The scientific community acknowledges the limitations of studying EDCs as individual compounds since in real life these chemicals exhibit a 'cocktail effect', however there is still a need to study these chemicals individually and assess their effects on specific tissues and reach a consensus on acceptable levels that do not have adverse effects. If the potency of individual chemicals is known, it becomes easier to influence legislation to monitor levels in the environment and manufactured goods.

1.10 Broad Aim

To investigate the effect of DBP on cell proliferation in the telencephalic regions of the brains of the adult male Japanese quail (C*oturnix coturnix japonica*) under the dosage regimes of low, medium high, over a 30 day pre-pubertal exposure to the phthalate ester, di-(n-butyl) phthalate (DBP).

1.10.1 Specific objectives

i) To assess the cytoarchitectural changes within the telencephalic regions of the brains of adult male Japanese quail (C*oturnix coturnix japonica)* following a 30 day pre-pubertal exposure to various doses of DBP, using Nissl staining.

ii) To assess the distribution of proliferating cell nuclear antigen immune-reactive cells (PCNA-ir) in telencephalic regions of the brains of the adult male Japanese

quail (C*oturnix coturnix japonica)* following pre-pubertal exposure to various doses of DBP.

iii) To assess the distribution of double cortin immuno-reactive cells (DCX-ir) within the telencephalic regions of the brains of the Japanese quail (C*oturnix coturnix japonica*), following pre-pubertal exposure to different doses of DBP.

iv) To quantify PCNA-ir cells within the ventricular zone (VZ) and medial pre-optic nucleus (POM) regions of Japanese quail (C*oturnix coturnix japonica)* brains following pre-pubertal exposure to various doses of DBP using imageJ.v1.460 image analysis software (National Institute of Health, USA).

v) To quantify DCX -ir within the hippocampus (Hp), mesopallium (M), nidopallium (N) and medial striatum (MSt) regions of Japanese quail (C*oturnix coturnix japonica)* brains following pre-pubertal exposure to various doses of DBP using imageJ.v1.460 image analysis software (National Institute of Health, USA).

2 CHAPTER 2: MATERIALS AND METHODS

2.1 Animals and housing

Newly hatched, male pre-sexed male Japanese quails (*Coturnix coturnix japonica*) sourced from the Aviary Unit Irene Animal Improvement Research Station, located in Pretoria, were used for the study. Prior to the experiment, the quail birds were immediately acclimatised in brooding cages at the Department of Production Animal Studies (Poultry Research Unit), University of Pretoria, for a period of two weeks, before being transferred into battery cages of dimensions: 49 x 95 x 51 cm. From hatch to four weeks of age, the temperature was adjusted by 0.5^{o}C/day, from 35-37^{o}C to 16-23^{o}C to achieve room temperature after a period of four weeks. The quail birds were maintained under a controlled photoperiod of 16L: 8D schedule of light/dark cycle which simulates long summer days (Bardet et al., 2012). Temperatures were maintained at 25±2^{o}C with a relative humidity of 50±5% until they attained the age of 10 weeks (SANS, 2008 guidelines). Throughout the experiment, the birds were fed a standard commercial high protein diet (Obaro Feeds, Pretoria, South Africa) and drinking water (tap water) was available *ad libitum*.

2.2 Chemicals and animal regime

DBP [CAS # 84-74-2], technical grade of 99% purity, was purchased from Sigma Aldrich, Johannesburg, South Africa, and used for the experimental dosing plan. DBP (the test compound) was dissolved in a vehicle of corn oil base and used for the treatment groups. The birds were randomly divided into four (4) dosage groups. The DBP control group was administered a corn-oil vehicle only (a dose of 1 mL/kg), while the other three experimental groups were administered intragastrically, using a daily dosage regimen of 10, 50, 400 mg DBP/kg/body mass, for a period of 30 days as depicted in Figure 2.1 (experimental scheme). Throughout the dosing period,

food intake, body weight and critical signs or any signs of abnormal behaviour were monitored daily. This study was part of an ongoing joint research study between the University of Pretoria, University of Witwatersrand and University of Science and Technology (NTNU), Trondheim, Norway. The standard protocols and approved experimental guidelines, according to research proposals of each University, were carried out. Overall, the experiment design was conducted according to the avian toxicity guidelines (OECD guidelines 2010). After the initial ethical approval, issued (vide No AEC/A058/12 [Amend] for the whole animal use, was obtained from the Animal Ethics Committee of the University of Pretoria, an ethical waiver [GF Dlamini Waiver 20-06-2017-O] was issued for the brain samples and research work undertaken at the University of the Witwatersrand.

2.2.1 Experimental design

The diagrammatic overview (Figure 2.1, below) depicts the schema for the experimental design. The guidelines for avian toxicity studies as stipulated by the Organisation for Economic Co-operation (OECD guidelines, 2010), was followed throughout the study.

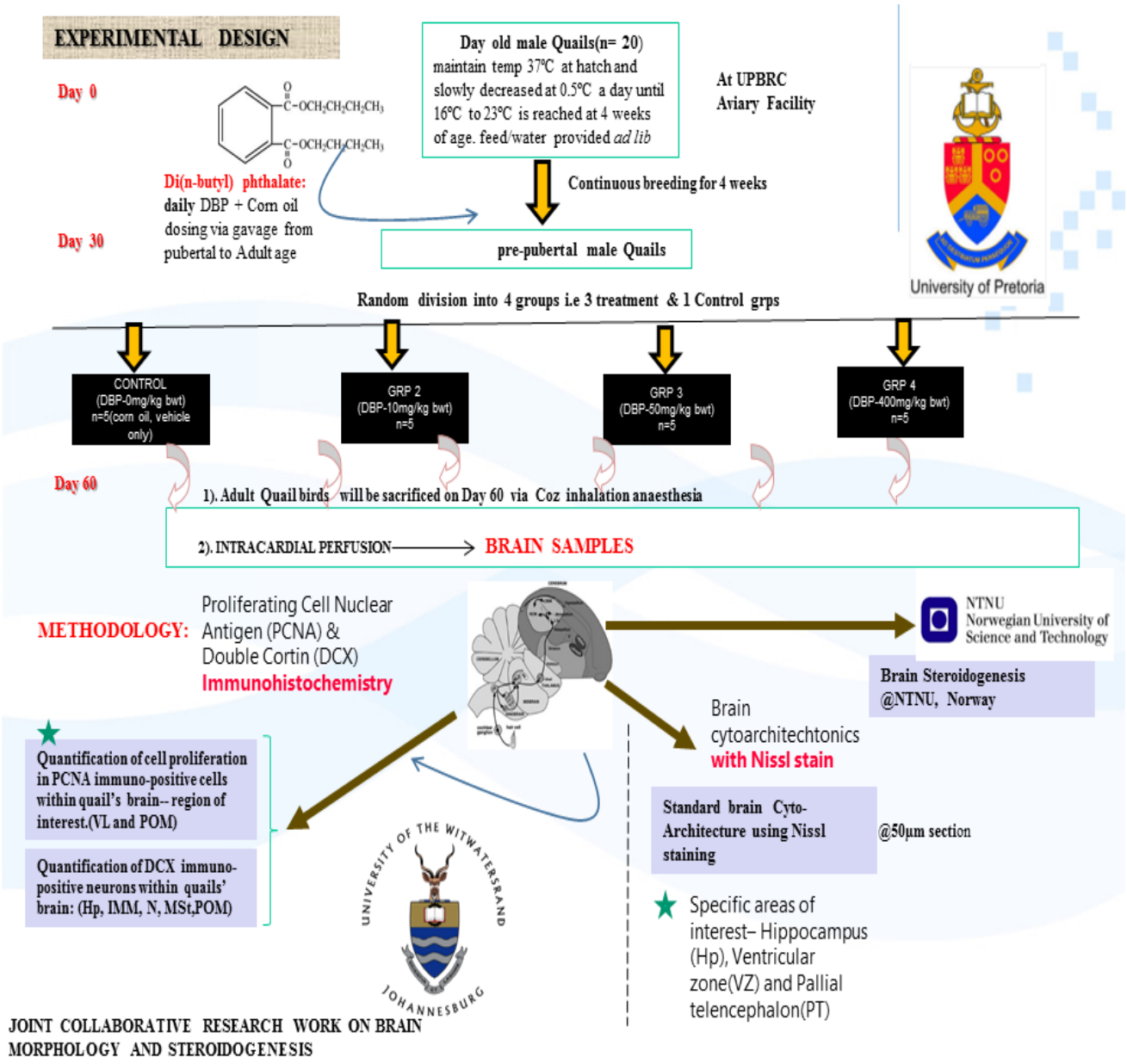


Figure 2.1: Schematic diagram showing the experimental design used for the present study.

2.3 Establishing a dosing protocol for di(n-butyl) phthalate (DBP) neurotoxicity studies in male Japanese quails (*Coturnix coturnix japonica*)

Several studies in laboratory animals have shown EDCs pose adverse health effects in humans and wildlife. Currently, there is no risk assessment data available on Non-Observable-Adverse-Effect-Levels [NOAEL] of DBP on fertility and neurological developments in avian species (US ATSDR, 2001); Bergman et al., 2013). However, there have been several attempts by various regulatory groups to estimate environmental concentrations of contaminants that should not cause harm to exposed wildlife (US ASTDR, 2001).

The first aim of the study was to design a dose-response relationship to DBP exposure and to further the neurological effects of a threshold dose of DBP on adult male Japanese quails. The estimates for human exposure to DBP range from 0.84 to 113µg/kg/day (Blount et al., 2000; Kohn et al., 2000). In a recent study on rodents, Kim and Jeon (2015) found DBP altered neurogenesis following prenatal exposure especially the middle-dose group of 50mg/kg, while Bello et al.'s (2014) avian study reported that DBP affected spermatogenesis in a dose and parameter dependant manner. Bello et al. (2014) consider both 200mg/kg and 400mg/kg/day, as high dose groups; while Kim and Jeon (2015), consider 10mg/kg and 50mg/kg as low dose and medium dose groups respectively. Overall, the doses used in the present avian study were chosen in order to achieve a dose-response relationship to DBP exposure on neurological effects using experimental low dose (10mg/kg body mass), medium dose (50mg/kg body mass) and high dose (400mg/kg body mass), in order to obtain a mechanistic view.

2.4 Perfusion and Fixation

After the 30 day experimental period, the quail birds were randomly selected from both control and experimental groups, and were euthanised using carbon dioxide (CO_2) inhalation anaesthesia. Birds were perfused transcardially with 0.9% cold saline solution followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB) according to the modified method of Salas et al. (1989). The brains were carefully removed from the skull and post fixed in 4% paraformaldehyde in 0.1M PB (pH 7.4) overnight. The whole quail birds were sacrificed at the University of Pretoria and the brains were harvested at the same time. Thereafter, the brains were transferred to the University of the Witwatersrand. The brains were transferred into small vial bottles containing 30% sucrose in 0.1M phosphate buffer and then stored at 4^{o}C until equilibrium was reached. They were then transferred to 1:1 mixture of ethylene glycol and glycerol in 0.244M PB (antifreeze) and stored at $^{-}20^{0}$C. Prior to sectioning, the brains were stored overnight in a solution of 30% sucrose in 0.1M PB.

2.5 Sectioning

Coronal sections were cut at 50µm in a rostro-caudal direction using a sliding microtome (Shandon Cryotome E and Cryomatrix gel Pittsburgh USA) on a freezing stage at -26^{0}C. Repeated five series sections were collected as free floating samples in individual wells and stored in 0.1M PB at 4^{0}C. The five series sections ensured that the periodicity of sampling was one section every 250µm (5x50µm=250µm) therefore sections were 250µm apart. This ensured that sampling was reproducible and homologous in the different experimental groups (Barker et al., 2014). However, sections could not be exactly matched to the quail brain (Bayle et al., 1974) atlas because no landmarks were implanted at stereotaxic landmarks on the live quail. Brains were trimmed in a rostro-caudal direction until the olfactory bulb

was visible which corresponds to level A14.00 according to the pigeon atlas of Karten and Hodos (1967), with the most caudal section demarcated by the anterior commissure (CA), which corresponds roughly to level A8.2 in the pigeon brain atlas. Both atlases were used because some coordinates of the quail brain are not available, secondly the system of coordinates used in the quail atlas were derived from the pigeon atlas (Bayle et al., 1974). One batch in the series of five sections was stained with the Nissl stain and two batches for doublecortin (DCX) and proliferating cell nuclear antigen (PCNA) immunohistochemical procedures.

2.6 Nissl staining

Free floating samples were mounted onto 0.5% gelatine coated slides and allowed to dry overnight. Slides were immersed in an alcohol/chloroform (1:1) defatting solution overnight then rehydrated in descending alcohol concentrations (100%:5 min; 95%:2 min; 70%:2 min; 50%:2 min), then placed in a cresyl violet stain for one minute, followed by distilled water for one minute. Samples were dehydrated again in 50% alcohol for 2 minutes and 70% alcohol where the optimal staining time was determined by stereomicroscopy. Dehydration was continued until 100% alcohol was reached. Slides were cleared in xylene and mounted with a Depex mounting medium (Merck chemicals (Pty) Ltd, Wadeville South Africa) and cover slipped. Cresyl violet stains Nissl bodies/RNA molecules blue.

2.7 Immunohistochemistry

Doublecortin (DCX) and Proliferating nuclear antigen (PCNA) were used as markers for cell proliferation and immature neuroblasts respectively. DCX is a neurotrophic microtubule associated phosphoprotein expressed by neuronal precursors, immature neurons and migrating neurons (Rao and Shetty, 2004). It is used as an

endogenous marker to visualise immature neurons and provide an estimate of neurogenesis that occurred within 20-30days of sacrifice (Chancellor et al., 2011; Kempermann, 2012). PCNA is a marker for the G1 and S phases of the cell cycle and is strongly associated with DNA replication sites in the cell nucleus (Kelman, 1997; Kurki et al., 1988). Antigen retrieval was performed for both PCNA and DCX. Individual sections were placed in Eppendorff tubes in a citrate buffer (pH 6). Eppendorff tube lids were pierced and placed on floating mats in a water bath at 80^0C for 30 minutes for antigen retrieval (Jiao et al., 1999). Sections were allowed to cool and rinsed three times with phosphate buffer (PB).

2.7.1 Immunohistochemistry for Doublecortin (DCX)

Free floating sections were kept at room temperature for 30 minutes followed by three 10 minute washes in phosphate buffer PB (0.1M) and transferred to an endogenous peroxidase inhibitor solution (EPI) containing 49.2 % methanol, 49.2 % PB and 1.6 % of 30% hydrogen peroxide (H_2O_2) by volume. The sections were kept in EPI for 30 minutes at room temperature under gentle agitation, followed by three 10 minute washes with PB (0.1M) solution. Thereafter, sections were incubated for 24 hours at 4^0C under gentle agitation in a rabbit polyclonal anti-DCX primary antibody (AB18723, Abcam; dilution 1:1000). Sections were rinsed three times for 10 minutes in PB (0.1M) and incubated further for 1-2 hours at room temperature in a biotinylated goat anti-rabbit secondary antibody (Vector Labs; dilution 1:500) in 3% normal horse serum and 2% granular bovine serum albumin (BSA). This was followed by three 10 minute rinses in PB (0.1M). Sections were further incubated in a DAB solution [3,3'-diaminobenzidene; 0.1MPB, 0.05% DAB, 0.01%H_2O_2] solution prepared 20 minutes prior to the procedure. Sections were soaked in 70% of the DAB solution for five minutes. Hydrogen peroxide was added to the remaining 30%

DAB solution and then added to the sections under gentle shaking. The DAB reaction results in a brownish colour which was monitored under a stereomicroscope until strong nuclear staining was apparent. Colour development was arrested by placing sections in 0.1M PB for 10 minutes followed by two 10 minute rinses in the same solution. Sections were mounted onto 0.5% gelatin coated slides and dried overnight. A graded series of alcohols was used for dehydration and slides were cleared in xylene and cover slipped with Depex® mounting medium.

2.7.2 Immunohistochemistry for Proliferating Cell Nuclear Antigen (PCNA)

Free floating sections were kept at room temperature for 30 minutes, followed by three 10 minute washes in phosphate buffer PB (0.1M) and transferred to an endogenous peroxidase inhibitor solution (EPI) containing 49.2 % methanol, 49.2 % PB and 1.6 % of 30% hydrogen peroxide (H_2O_2) by volume, kept for 30 minutes at room temperature under gentle agitation, followed by three washes of 10 minutes with PB (0.1M) solution. Thereafter, sections were incubated for 24 hours at 4^0C under gentle agitation in a mouse monoclonal anti-PCNA primary antibody (AB (PC10) a29, Abcam; dilution 1:500). Sections were rinsed three times for 10 minutes in PB (0.1M) and incubated further for 1-2 hours at room temperature in a biotinylated horse anti-mouse secondary antibody (Vector Labs; dilution 1:500) in 3% normal horse serum and 2% granular bovine serum albumin (BSA).

This was followed by three 10 minute rinses in PB (0.1M). Sections were further incubated in DAB (3,3'-diaminobenzidene; 0.1MPB, 0.05% DAB, 0.01%H_2O_2) solution prepared 20min prior to the procedure. Sections were soaked in 70% of the DAB solution for five minutes. Hydrogen peroxide was added to the remaining 30% DAB solution and then added to the sections under gentle shaking. The DAB

solution reaction results in a brownish colour which was monitored under a stereomicroscope until strong nuclear staining was apparent. Colour development was arrested by placing sections in 0.1M PB for 10 minutes followed by two 10minute rinses in the same solution. Sections were mounted onto 0.5% gelatin coated slides and dried overnight. A graded series of alcohols was used for dehydration and slides were cleared in xylene and cover slipped with Depex® mounting medium.

Table 2.1: Primary and Secondary antibodies used in this study

Primary antibodies	**Secondary Antibodies**	**Normal serum Secondary**
Anti-PCNA mouse monoclonal 1:500 (Abcam)	Biotinylated horse anti-mouse 1:1000 (Vector Labs)	Normal horse serum
Anti-DCX rabbit polyclonal 1:1000 (Abcam)	Biotinylated goat anti-rabbit 1:1000 (Vector Labs)	Normal goat serum

2.8 Data Analysis

2.8.1 Qualitative Analysis

Neuroanatomical borders were outlined using a stereomicroscope and Nissl stained slides to map out the ventricular zones (VZ), hippocampus (Hp), pallial telencephalon, olfactory bulb, medial striatum (Mst), and medial preoptic nucleus (POM).

Brain structures were identified according to the stereotaxic description of the quail or pigeon brain atlas (Karten and Hodos, 1967; Bayle et al.,1974). Both atlases were used because some coordinates of the quail brain are not available, secondly

the system of coordinates used in the quail atlas were derived from the pigeon atlas (Bayle et al., 1974). Antero-posterior sections taken at approximately 8.2 level as described in the pigeon stereotaxic atlas by Karten and Hodos (1967) were surveyed using a Leica DMRB microscope (Leica Microsystems) to identify and map out DCX and PCNA immuno-reactive cells (DCX-ir/PCNA-ir).

2.8.2 Quantitative Analysis

Following a qualitative survey to identify immuno-reactive areas for DCX, the cells were systematically quantified in four principal brain areas. The chosen areas have a possibility of varying DCX expression as reported by previous studies (Vellema et al., 2013; Melleu et al., 2013). Full cellular outlines of DAB labelled DCX-ir cells could not be fully deciphered, however cells with stained cytoplasm and an unstained nucleus with or without stained neurites were counted (Balthazart et al., 2008) at three different rostro-caudal levels in sections that are 50 µm apart, at rostral, intermediate, and caudal levels with the intermediate section being the starting point corresponding to plate A 8.2 or plate 12 of chicken brain atlases (Kuenzel and Masson,1988). DCX-ir were quantified in the four brain regions using the software imageJ.v1.460 (National Institute of Health USA). Images were digitally acquired by video camera (DX 720, Olympus camera) attached to a microscope (Olympus BH-2, 40x objective) connected to a Macintosh computer with the image analysis software from ImageJ (National Institute of Health USA). Cells were counted manually by the investigator and one researcher who was blind to the different treatment groups. Cells were counted in the photomicrographs in a 536 x 360 µm field in landscape at each of the rostro caudal sections placed on predetermined anatomical landmarks like ventricles and fibre tracts as described in

quail, pigeon and chicken atlases (Karten and Hodos, 1967; Bayle et al., 1974; Kuenzel and Masson 1988).

Cells were counted on either right or left hemispheres randomly chosen with predetermined anatomical structures as guides (Bardet et al., 2012). When multiple fields were chosen per section at the same rostro caudal level, the data was totalled before any further analysis. The cell count approach for each brain region of interest is indicated below.

Hippocampus (Hp)

The quantification field was aligned between the inter hemispheric fissure and lateral ventricle (Taziaux et al., 2007) measuring 536 x 360 µm camera field for all three rostrocaudal sections corresponding to plate A 8.2 of the chicken brain atlas by Kuenzel and Mason (1988). Counts were grouped as Hp (R1,R2,R3) for all treatment groups (10mg 50mg, 400 mg) plus control (Figure 2.1)

Intermediate Medial Mesopallium (IMM)

Two adjacent camera fields were used to quantify cells within the IMM from three rostro-caudal sections corresponding to plate A 8.2 in the chicken brain atlas of Kuenzel and Masson (1988) with the intermediate section being the starting point. The first field was demarcated ventrally by the mesopallial lamina and laterally by the edge of the lateral ventricle (Taziaux et al., 2007).

The second field was one length (536 µm) along the lateral ventricle above the first field. Counts were grouped as MM1 (R1, R2, R3) and MM2 (R1, R2, R3) for all treated groups plus control (Figure 2.1).

Nidopallium Intermedium (N)

Three rostro-caudal sections 50 µm apart corresponding to plate A 8.2m according to Kuenzel and Mason (1988), were used for quantification. The camera field was initially located at the edge of the lateral ventricle where the ventricles are closest to the midline. The second field located one width (360 µm) laterally to the centre of the nidopallium. The last field placed further laterally almost touching the edge of the brain section (modified from Taziaux et al., 2007; Nikolopolou et al., 2006). Counts were grouped as N1 (R1, R2, R3); N2 (R1, R2, R3); N3 (R1,R2, R3) for all groups plus control (Figure 2.1).

Medial Striatum (MSt)

The camera field was located above the lateral bed nucleus of the stria terminalis (BSTL) at the edge of the lateral ventricle. Counts were done at all three rostro-caudal sections starting with intermediate section located at plate A 8.2 (Kuenzel and Masson, 1988). Counts were grouped as MSt (R1, R2, R3) for all treated groups and control (Figure 2.1).

Medial Preoptic Nucleus (POM)

PCNA-ir cells were counted at three rostro-caudal sections of the POM at the most caudal part of the medial preoptic area (mPOA) where the anterior commissure crosses the midline and reaches its largest extension.

This area roughly corresponds to plate A 8.2 as described by Kuenzel and Masson (1988). The camera field was aligned with the lateral edge of the 3rd ventricle and ventral edge of the anterior commissure (Taziaux et al., 2007; Bardet et al., 2012).

Counts were grouped as POM (R1, R2, R3) for all treated groups plus control (Figure 2.1).

Telencephalic Ventricles (VL)

A superficial ventricular zone boundary was demarcated by a sharp decrease in DAB staining and anti-PCNA staining which was roughly within 30 µm of the inner walls of the lateral ventricles. The camera field was placed at the apex of either right or left ventricles with the hippocampus forming the medial wall. The counting area spanned the full length of the camera field (536 µm x 30 µm) (modified from Charvet and Striedter, 2008; Barker et al., 2014).

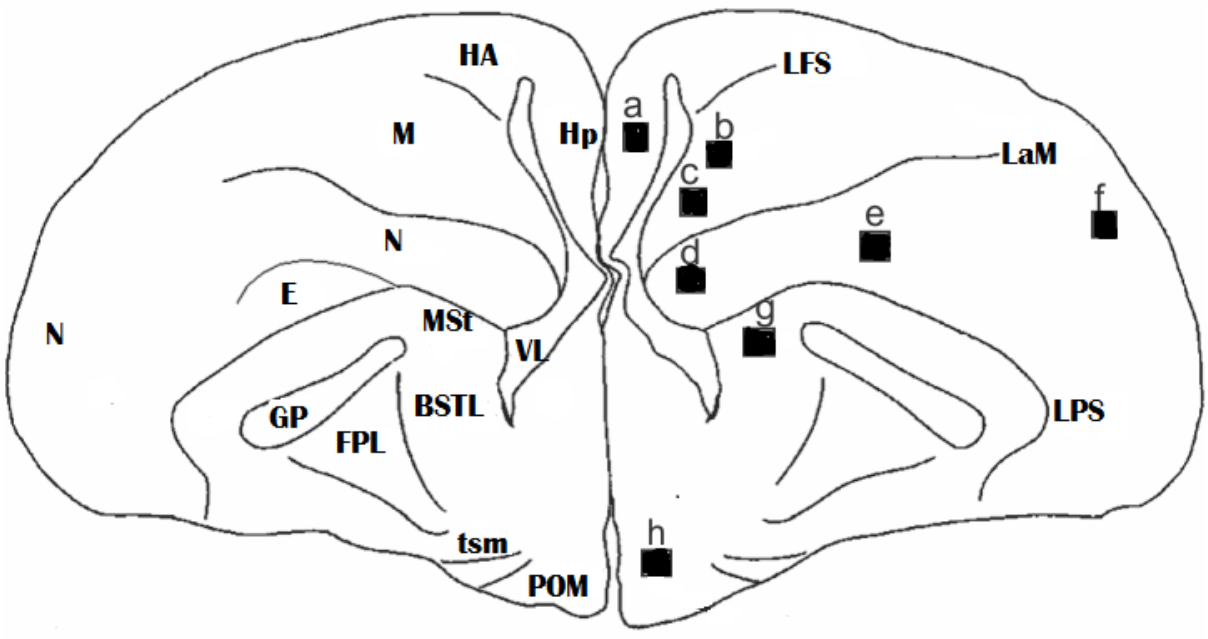


Figure 2.2: Schematic diagram showing landmarks that delineate various areas of the Japanese quail brain coronal section (modified from Bardet et al., 2012). This image is copied in terms of "fairdealing" in terms of section 12 of South African copyright act of 1978 (as amended).

The squares labelled **a** to **h** represent areas of quantification. The box labelled ' **a'** is located in the hippocampus that lies between the inter-hemispheric fissure and lateral ventricle (VL), **'b**' and **' c**' are located in the mesopallium (M) as two fields along the lateral ventricles, where the mesopallium is delineated by the LaM ventrally. Boxes '**d, e** and **f**' are the three quantification fields in the mesopallium, '**d**' is located close to the edge of the lateral ventricle, '**e**' towards the centre of the mesopallium, with '**f**' located towards the periphery of the mesopallium. '**g**' represents the medial striatum (Mst) at the edge of the lateral ventricle above the lateral bed nucleus of the stria terminalis (BSTL). '**h**' represents the medial pre-optic nucleus (POM). E, entopallium; FPL, lateral forebrain bundle; GP, globus pallidus; HA, hyperpallium Apicale; Hp, hippocampus; LaM, lamina mesiopallialis; LFS, lamina frontalis superior; LPS, lamina pallio-subpallialis; LSt, lateral striatum; M, mesopallium; N, nidopallium; TSM, tractus septopalio-mesencephalicus.

2.9 Statistical Analysis

Data obtained in the experiment was analysed using Stata/IC version 14.1 for each category of dosage, region, and rostrocaudal levels of DCX positive cells and PCNA positive cells. An SK (Skewness-Kurtosis) test was performed to check for normal distribution of the response variable. Differences between the three rostro-caudal levels were analysed with one way analysis of variance (ANOVA). Differences in counts between treated groups and the control, as well as differences between the individual groups were analysed using linear regression. Data obtained was averaged and expressed as a mean ± standard deviation (SD). Reported cell counts may have been over-estimated as double counting was not accommodated. However the size of DCX-ir and PCNA-ir nuclei (4-7 µm) or counted objects is much smaller than section thickness (50 µm) which eliminates overestimation due to double counting. According to the Abercrombie (1946) correction, numbers of positively identified objects should be inflated by 10% (N=n*(30/(30+3), where N=corrected number and n=observed number to accommodate double counting. This ratio is not affected because the size of counted objects does not change per group.

3 CHAPTER 3: RESULTS

3.1 Brain morphology

The harvested Japanese quail brains used in this study exhibit a typical avian brain with all major brain areas visible to the naked eye for those sections that were intact. Easily discernible brain areas were the telencephalon, midbrain, and cerebellum. When viewed in a rostro-caudal direction, and based on the measurements in Figure 3.1, the quail brain depicts a larger telencephalon, a smaller midbrain, and more elongated cerebellum.

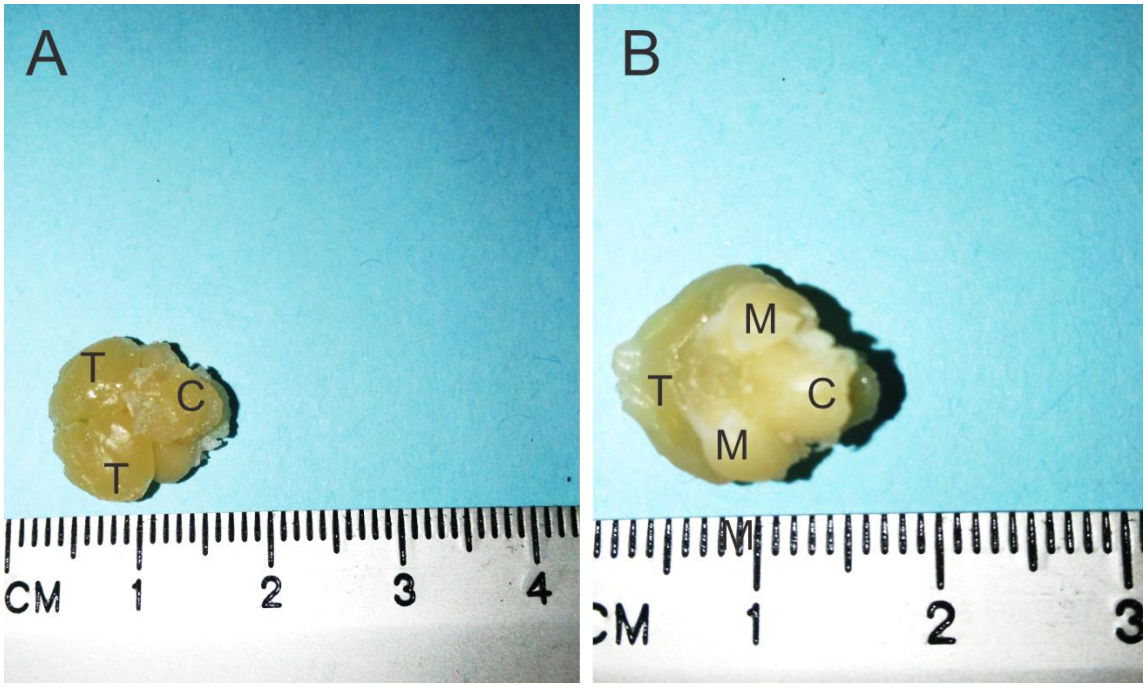


Figure 3.1: Photographs showing A, the dorsal view and B showing the ventral view of the Japanese quail (Cortunix coturnix japonica) whole brain. C-cerebellum; M-midbrain; T-telencephalon.

3.2 Cytoarchitecture of the quail brain using Nissl stain Histology

Coronal Nissl stained sections were used for the identification of brain areas in conjunction with several brain atlases to study the brain cytoarchitecture in specific areas of interest (Figure 3.2). Coronal sections obtained roughly at level A 14.00, according to Karten and Hodos (1967), showed the olfactory bulb with an increased cellular density along its ventricle and a less intense staining towards the periphery. Sections corresponding roughly to level A 8.25, according to Karten and Hodos (1967), displayed a typical avian brain with most of the areas of interest being easily discernible, namely the ventricles, hippocampus, nidopallium, mesopallium, medial striatum and medial pre-optic nucleus. The superior frontal lamina (LFS) separating the mesopallium from the hippocampus and mesopallial lamina (LaM) separating the mesopallium from the nidopallium were easily discernible as lighter staining lines from the periphery towards the midline.

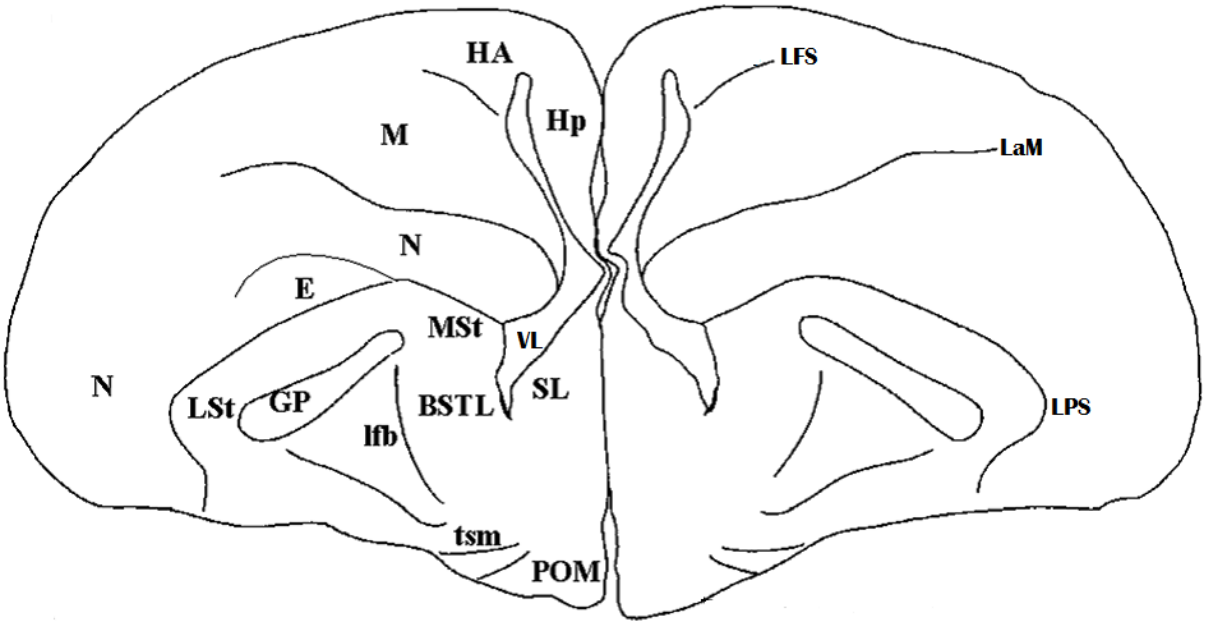


Figure 3.2: Schematic diagram showing landmarks that delineate various areas of the Japanese quail brain coronal section (modified from Taziaux et al., 2007). This image is copied in terms of "fairdealing" in terms of section 12 of South African copyright act of 1978 (as amended).

BSTL, lateral bed nucleus of the stria terminalis; **E**, entopallium; **lfb**, lateral forebrain bundle; **GP**, globus pallidus; **HA**, hyperpallium Apicale; **Hp**, hippocampus; **LaM**, lamina mesiopallialis; **LFS**, lamina frontalis superior; **LPS**, lamina pallio-subpallialis; **LSt**, lateral striatum; M, mesopallium; MSt, medial striatum; N, nidopallium; POM, medial preoptic nucleus; **SL**, lateral septal nucleus; **tsm**, tractus septopalio-mesencephalicus; **VL**, lateral ventricle.

A gradual reduction in cellular densities from the control to the high dose group of 400 mg was observed, with the medium dose and low dose exhibiting similar cellular densities (figure 3.3). Intense cellular densities were observed along the lateral ventricles and third ventricle with a gradual decline in ependymal cell layers from the control to the high dose group (figure 3.4). Fibres of the anterior commissure crossing the midline could be identified with densely stained large cells of the POM immediately ventral to the anterior commissure (figure 3.5). The medial striatum at the tips of the lateral ventricles just above the BSTL had more intense staining and increased cellular densities.

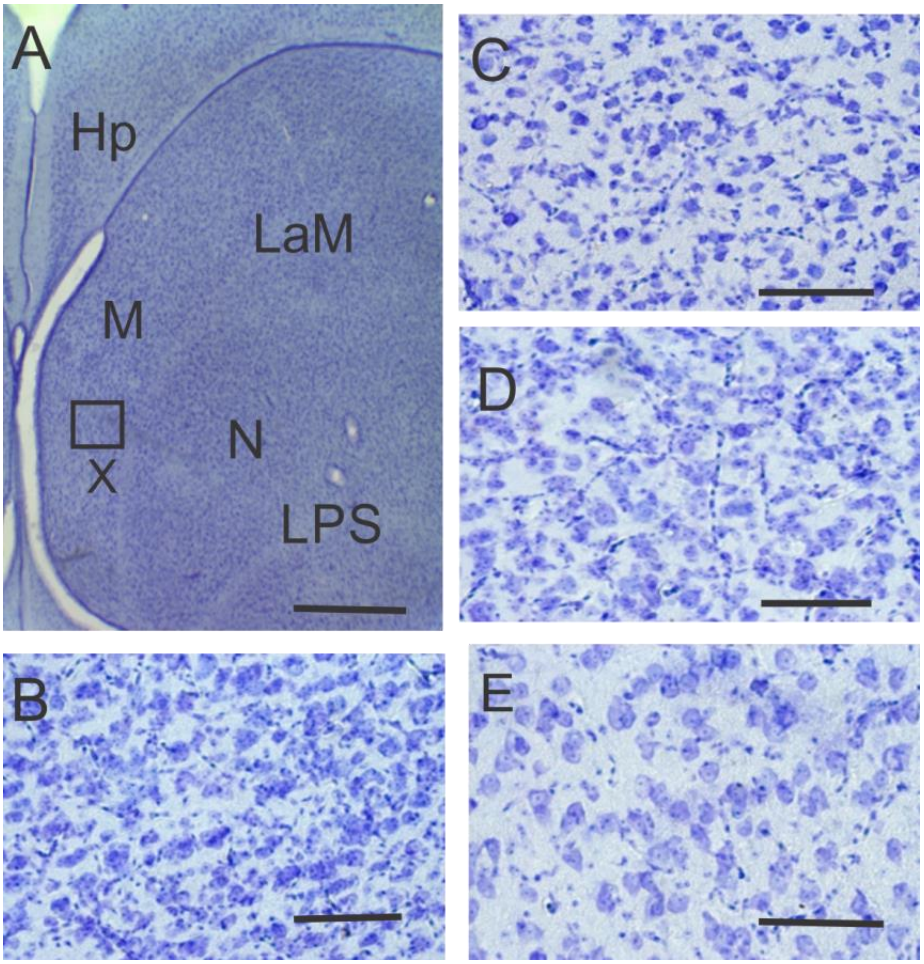


Figure 3.3: Light microscopy photomicrographs of the adult male Japanese quail (Coturnix coturnix japonica) brains following a 30-day pre-pubertal exposure to DBP.

Figure **A** is the left hemisphere of the DBP control brains, with well demarcated anatomical regions. **Figures B, C, D** and **E** represent a higher magnification of the area marked X in A. **Figure B** represents the DBP control whilst **Figures B, C, D** and **E** represent the different DBP doses of low (10 mg), medium (50 mg) and high (400 mg) respectively. Note there is a gradual reduction in cellular densities from the control to the high dose of 400 mg. Hp, hippocampus; LaM, lamina mesiopallialis; LPS, lamina pallio-subpallialis; M, mesopallium; N, nidopallium; VL, lateral ventricles. Scale bar: **A** =X 100; **B**, **C, D** and **E** = X400. Nissl stain.

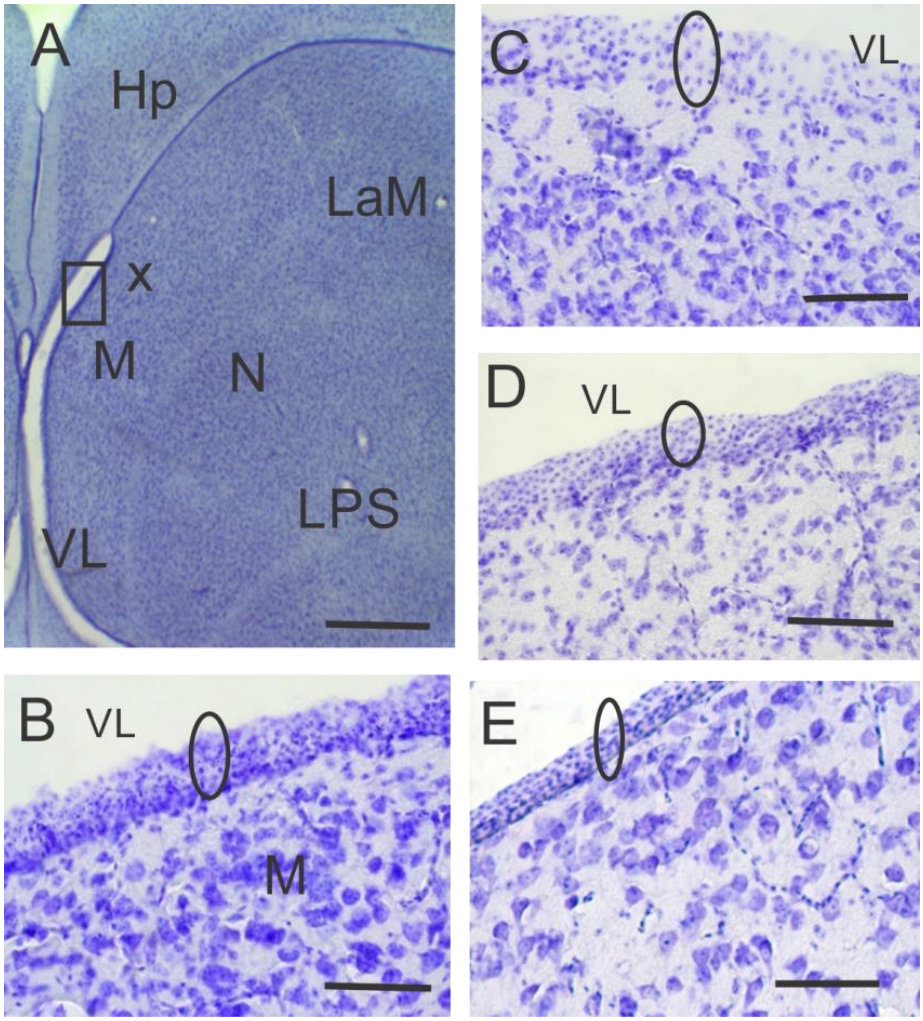


Figure 3.4: Light microscopy photomicrographs of the adult male Japanese quail (Coturnix coturnix japonica) brains following a 30-day pre-pubertal exposure to DBP.

Figure A shows the lateral ventricles (VL), the lighter staining LaM (lamina mesopallialis**)** and LPS (lamina pallio-subpallialis) separating the mesopallium (M) and nidopallium (N). **Figures B, C, D** and **E** represent the mesopallium (M) adjacent to the ependymal layer marked with an X in **Figure A**. The ellipses in **B, C, D** and **E** indicate the layers of ependymal cells and neurogenic cells lining the ventricle lumen (VL). The DBP control (**B**) and low dose(**C**) groups appear to have a thicker layer of cells compared to the DBP medium(**D**) and DBP high dose(**E**) group. Hp, hippocampus. Scale bar: **A** =X 100; **B**, **C**, **D** and **E** = X400. Nissl stain.

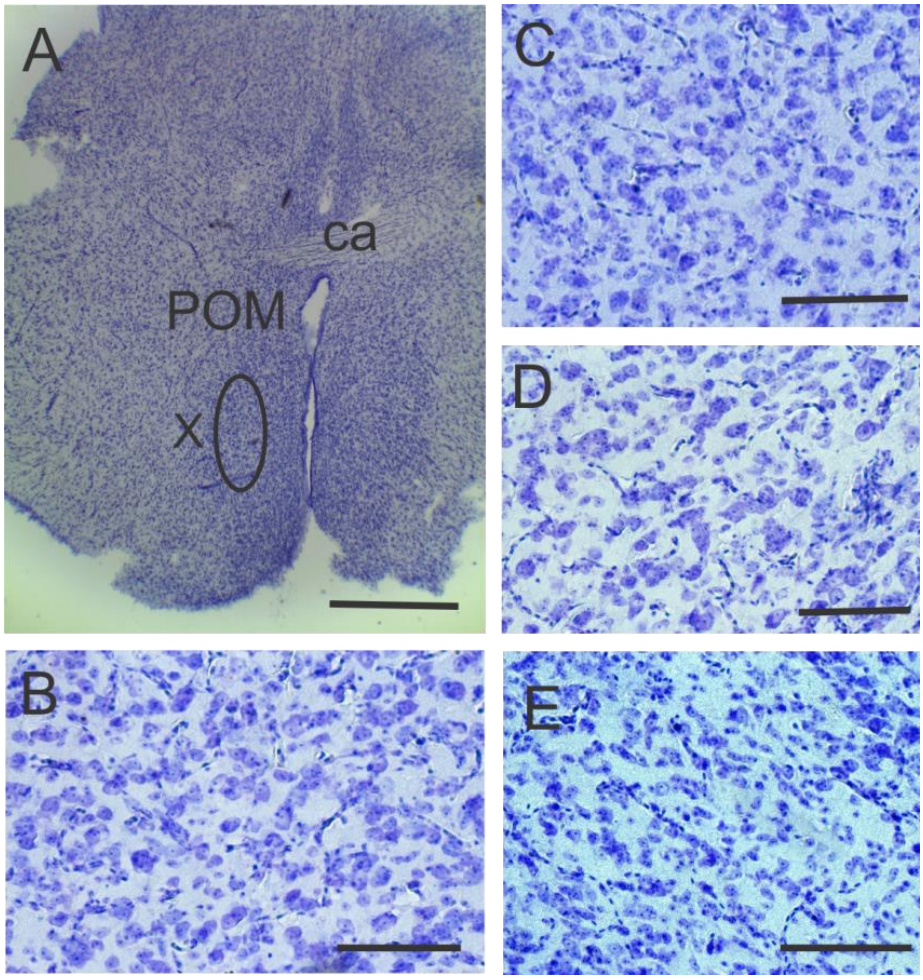


Figure 3.5: Light microscopy photomicrographs of the adult male Japanese quail (Coturnix coturnix japonica) brains following a 30-day pre-pubertal exposure to DBP.

Figure A represents the medial pre-optic nucleus (POM) below the anterior commissure (ac). The elliptical insert marked X represents the quantification area for **Figures B, C, D** and **E**. **Figure B** is the control, **C** low dose (10 mg), **D** medium dose (50 mg) and **E** high dose (400 mg) respectively. Note the cellular densities appear similar in the DBP control (**B**), DBP low dose(**C**) and DBP medium dose (**D**) with a slight reduction in the DBP high dose (**E**) groups. Scale bar: **A** = X100; **B**, **C**, **D** and **E** = X400. Nissl stain.

3.3 Immunohistochemistry

DCX labelling was predominantly on cell bodies with very sparse or no staining at all on neurites, especially with the treatment groups making quantification of the different types of neurons (round, fusiform, multipolar) impossible. Only cells with cytoplasmic staining and an unstained nucleus, with or without stained neurites, were counted. DCX-ir cells were counted in the hippocampus, medial mesopallium, nidopallium, and medial striatum. PCNA-ir cells were counted in the POM and telencephalic ventricles.

3.3.1 Distribution of DCX-ir cells

Various areas of the telencephalon showed concentrations of DCX-ir cells which were strongest along the ventricles and tended to fade as one moved away from the ventricles. These cells were scattered within the hippocampus, hyperpallium and entopallium with increased densities especially within the mesopallium, medial stritatum and olfactory bulbs. Within the nidopallium, increased concentrations were observed within areas that are proximal or closest to the midline and distal or towards the edge of the section with the intermediate part showing lighter densities (figure 3.6). The control showed stronger cellular densities compared to the three treatment groups, however there were no discernible differences between the treatment groups.

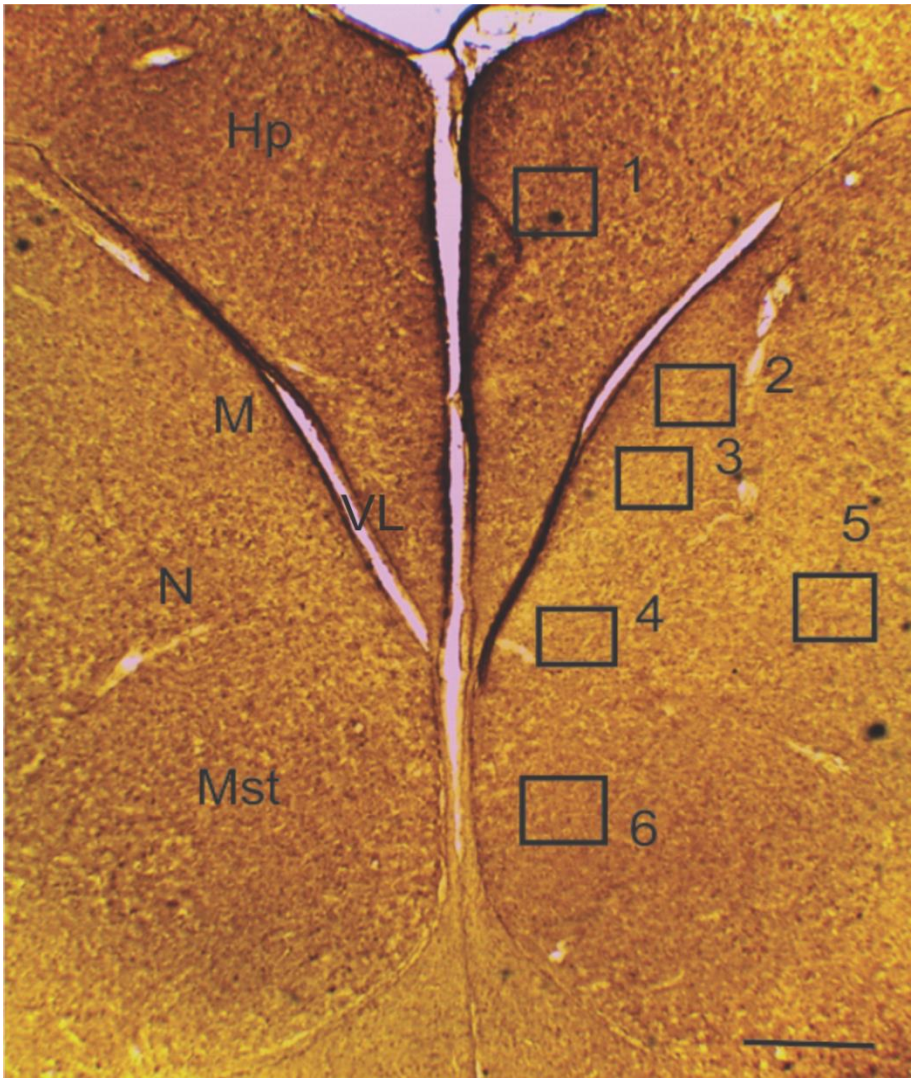


Figure 3.6: Light microscopy photomicrographs of the adult male Japanese quail (Coturnix coturnix japonica) brains following a 30-day pre-pubertal exposure to DBP.

Numbered boxes show the quantification areas in the hippocampus (1) mesopallium (2,3), nidopallium (4,5), and medial striatum (6). Increased cellular densities along the ventricles (VL) can be observed. Scale bar =X100. DCX stain.

3.3.2 Distribution of PCNA-ir cells

PCNA-ir were most abundant in the ventricular zones of the telencephalon, olfactory bulb, periventricular zones of the 3rd ventricle and ventricular zones adjacent to the hippocampus, mesopallium, nidopallium, medial striatum and the POM (figure 3.7).

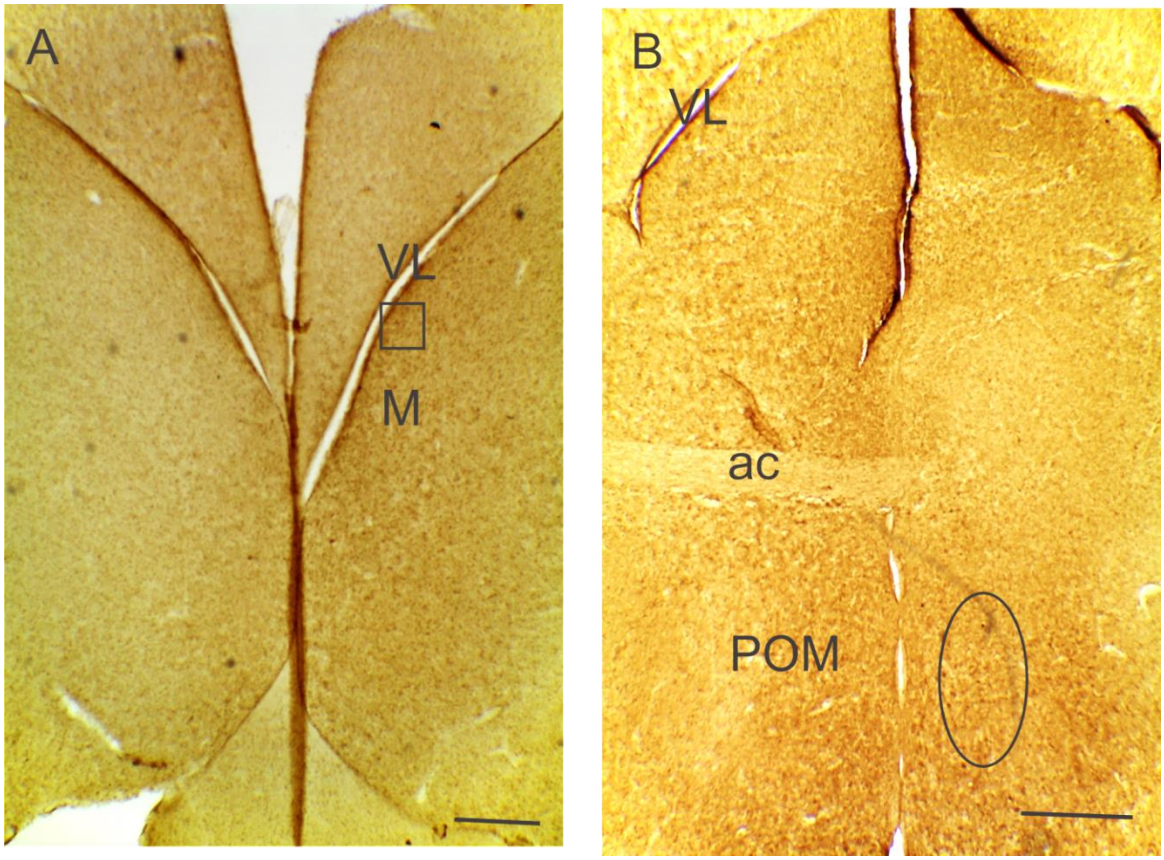


Figure 3.7: Light microscopy photomicrographs of the adult male Japanese quail (Coturnix coturnix japonica) brains following a 30-day pre-pubertal exposure to DBP.

The square in **A** represents the quantification area within the ventricles located above the mesopallium (M), while the ellipse in **B** represents the quantification area in the medial pre-optic nucleus (POM). ac, anterior commissure; VL, lateral ventricle. Scale bar: **A** and **B** = X100. PCNA stain.

3.3.3 Numbers of DCX-ir cells

Cells showing DCX immunoreactivity were characterised by cytoplasmic staining with minimal neurite staining or no neurite staining. Representative photomicrographs of the five brain regions showing DCX-ir cells are shown in figure 3.9, figure 3.10, figure 3.11 and figure 3.12. Summary statistics for the response variable and exposures showed a normal distribution when subjected to an SK test (figure 3.12).

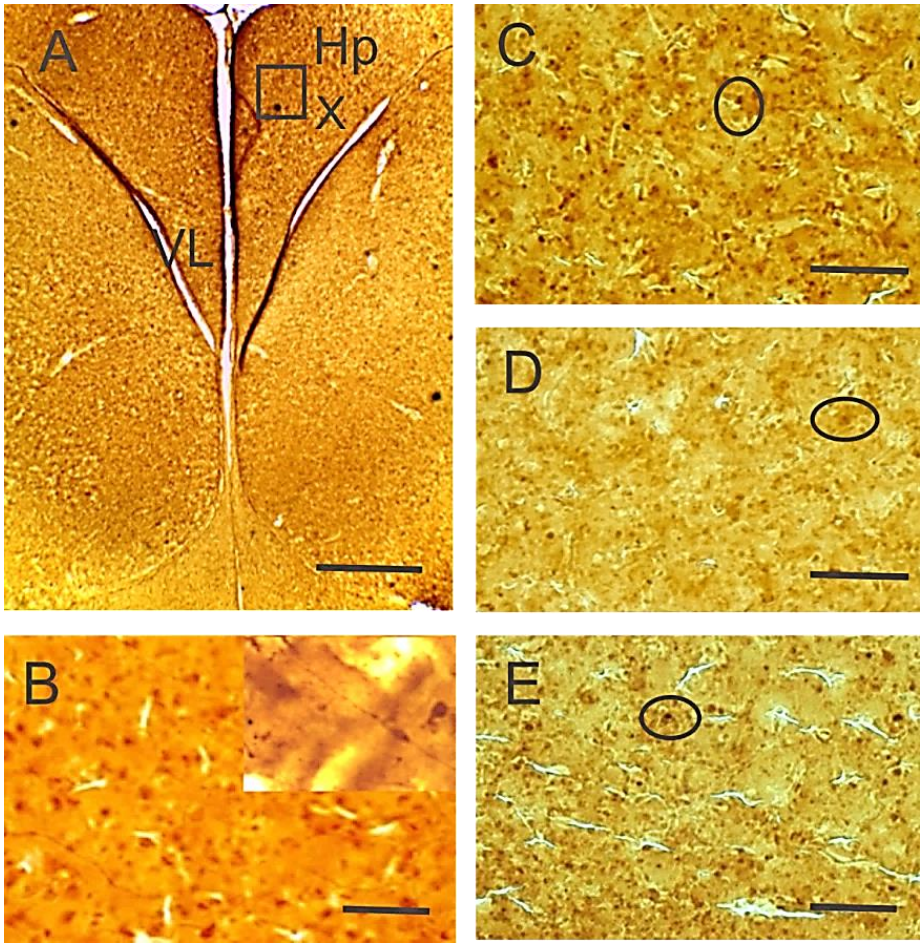


Figure 3.8: Light microscopy photomicrographs of the adult male Japanese quail (Coturnix coturnix japonica) brains following a 30-day pre-pubertal exposure to DBP.

The square marked X in **A** indicates the quantification field within the Hippocampus (HP). photomicrographs **B, C, D** and **E** represent the DBP control, low (10mg), medium, (50mg) and high (400mg) dose groups respectively. The insert in **B** shows a bipolar DCX positive cell. Ellipses within **C**, **D** and E indicate some DCX positive cells. Higher cellular densities within the low dose(**C**) compared to the medium (**D**) and high doses (**E**) can be observed. VL, ventricles. Scale bar: A = X100, B, C, D and E = X400, insert in B = X630. DCX stain.

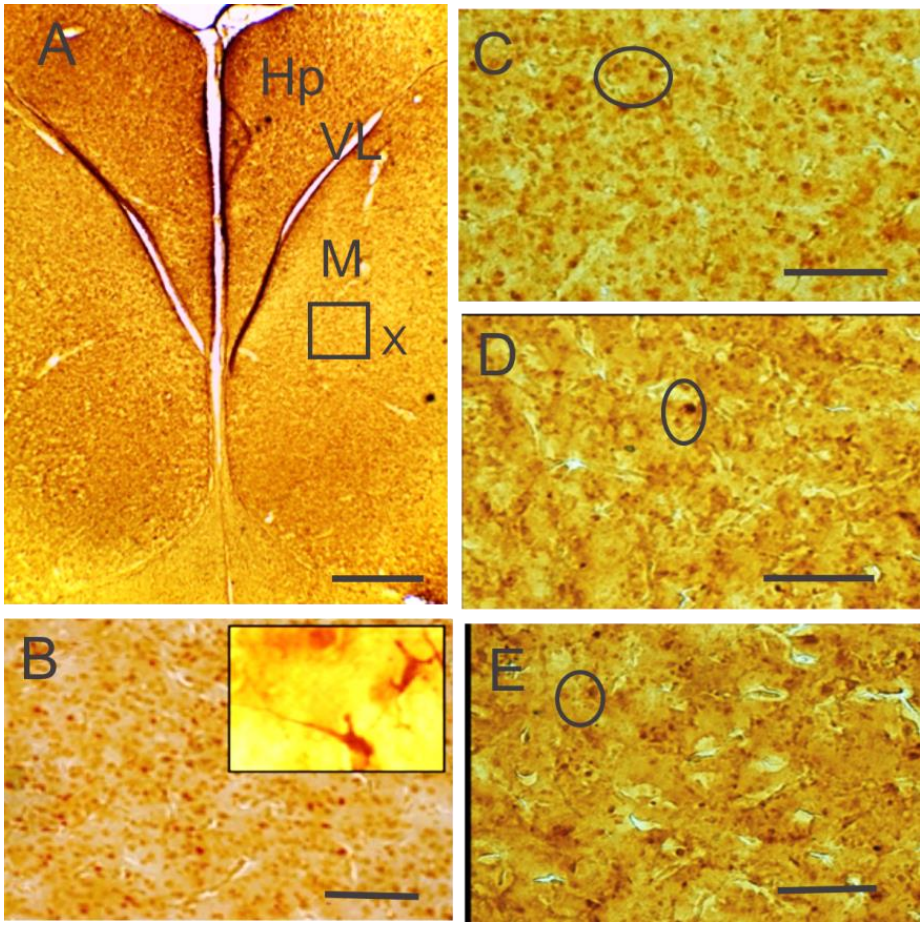


Figure 3.9: Light microscopy photomicrographs of the adult male Japanese quail (Coturnix coturnix japonica) brains following a 30-day pre-pubertal exposure to DBP.

The square marked X in **A** indicates the quantification field within the Mesopallium (M). Photomicrographs **B, C, D** and E represent the DBP control, low (10mg), medium, (50mg) and high (400mg) dose groups respectively. The insert in **B** shows bipolar and multipolar DCX positive cells. Ellipses within **C**, **D** and **E** indicate some DCX positive cells. VL, ventricles; Hp, Hippocampus. Scale bar: A = X100, **B**, **C**, **D** and **E** = X400, insert in **B** = X630. DCX stain.

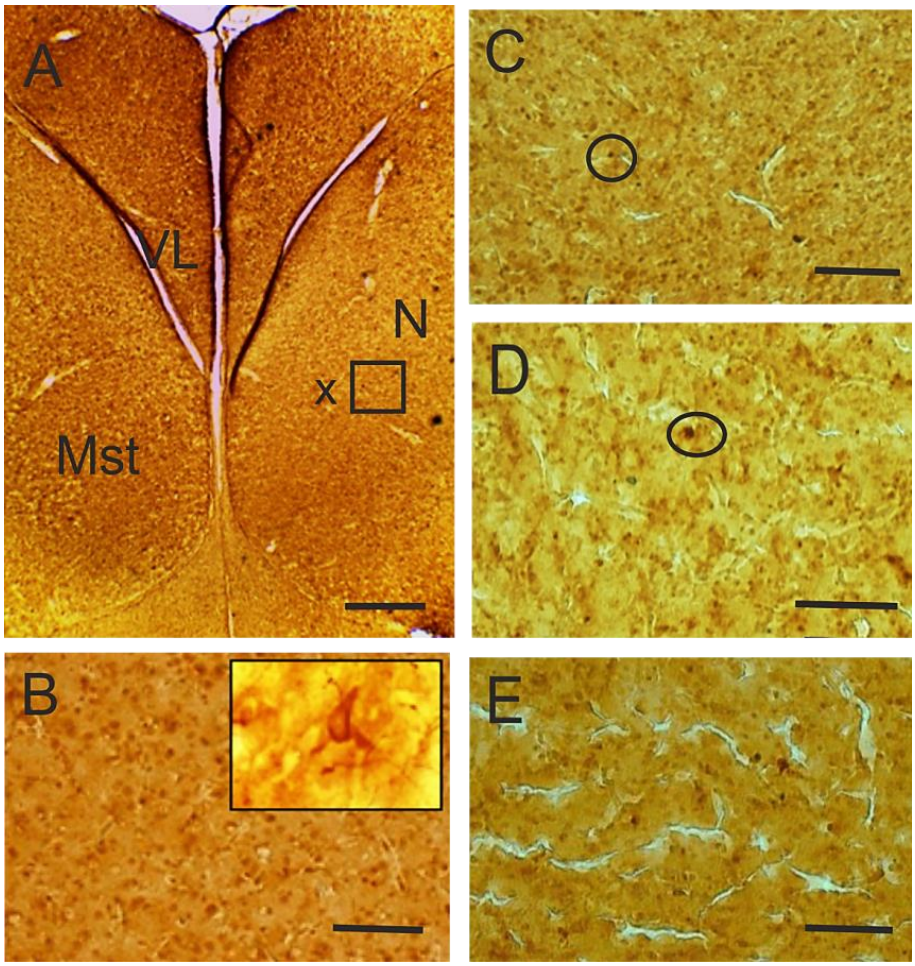


Figure 3.10: Light microscopy photomicrographs of the adult male Japanese quail (Coturnix coturnix japonica) brains following a 30-day pre-pubertal exposure to DBP.

The square marked X in **A** indicates the quantification field within the nidopallium (N). Photomicrographs **B**, **C**, **D** and **E** represent the DBP control, low (10mg), medium, (50mg) and high (400mg) dose groups respectively. The insert in **B** shows a multipolar DCX positive cell. Ellipses within **C** and **D** indicate some DCX positive cells. Higher cellular densities within the control (**B**) and low dose (**C**) compared to the medium (**D**) and high doses (**E**) can be observed. VL, ventricles; MSt, medial striatum. Scale bar: **A** = X100, **B**, **C**, **D** and **E** = X400, insert in **B** = X630. DCX stain.

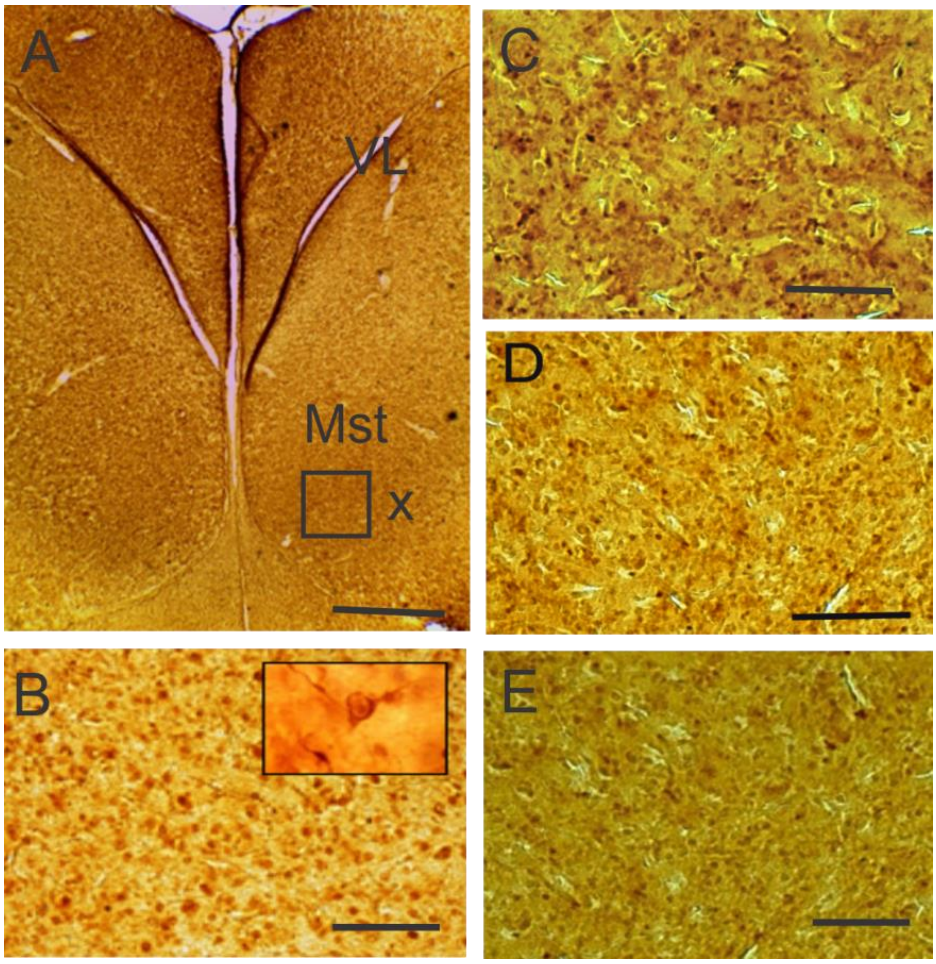


Figure 3.11: Light microscopy photomicrographs of the adult male Japanese quail (Coturnix coturnix japonica) brains following a 30-day pre-pubertal exposure to DBP.

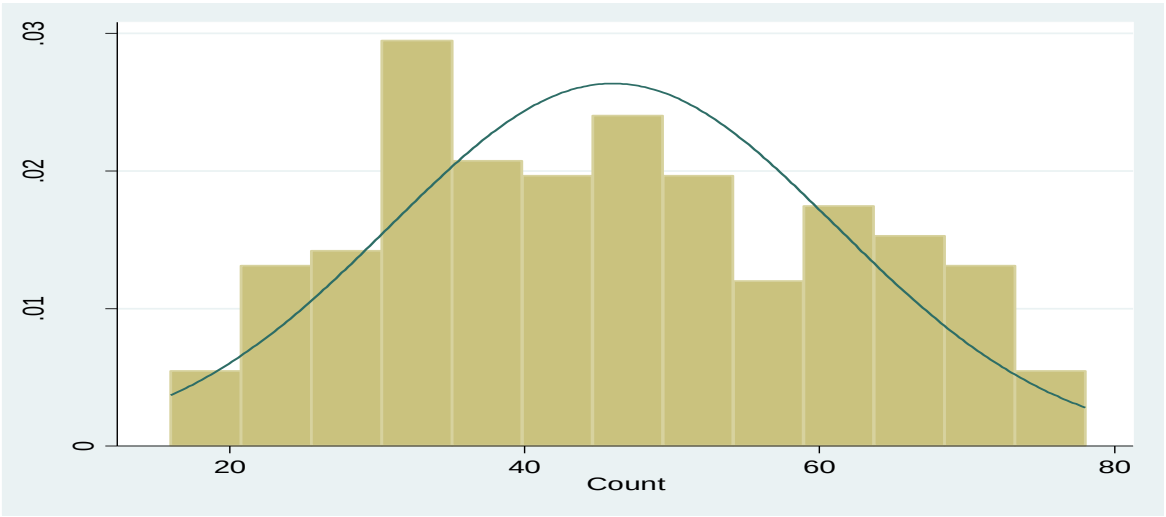
The square marked X in **A** indicates the quantification field within the medial striatum (MSt). Photomicrographs **B**, **C**, **D** and E represent the DBP control, low (10mg), medium, (50mg) and high (400mg) dose respectively. The insert in **B** shows a multipolar DCX positive cell. VL, ventricles. Scale bar: **A** = X100, **B**, **C**, **D** and **E** = X400, insert in **B** = X630. DCX stain.

Systemic counts of DCX-ir cells in each dosage group for the five brain areas were performed. The data is summarised in table 3.1. Counts ranged between 18 and 75 with the highest count observed in the control group (68.17±6.46) of the IMM. In the low dose group (10mg). the medial striatum had the lowest count (30±10.1) while the IMM had the highest count (40.25 ±11.87). In the medium dose group (50mg), the POM recorded the highest mean count of 51.50±12.55, with the lowest being in the hippocampus (37.17 ±11.32). Within the high dose group (400mg), the medial striatum had the highest count (50.17 ±10.87) with the nidopallium recording the lowest count (37.44±6.92). Figure 3.12 represents counts in the various brain areas presented as a bar graph.

Table 3.1: Summary of DCX-ir cell numbers in different brain regions in the adult male Japanese quail brain expressed as mean ± standard deviation of the mean.

	Brain Anatomical Regions				
Dose (mg/kg/day)	Hp	IMM	N	POM	MSt
400	38.83(12.35)	46.08(12.65)	37.44(6.92)	38.67(10.75)	50.17(10.87)
50	37.17(11.32)	39.17(13.42)	40.61(13.38)	51.5(12.55)	39.83(7.36)
10	36.17(11.57)	40.25(11.87)	36.94(9.67)	35(6.96)	30(10.1)
Control	66(6.56)	68.17(6.46)	63.89(7.22)	60(8.19)	64.33(8.14)

A



B

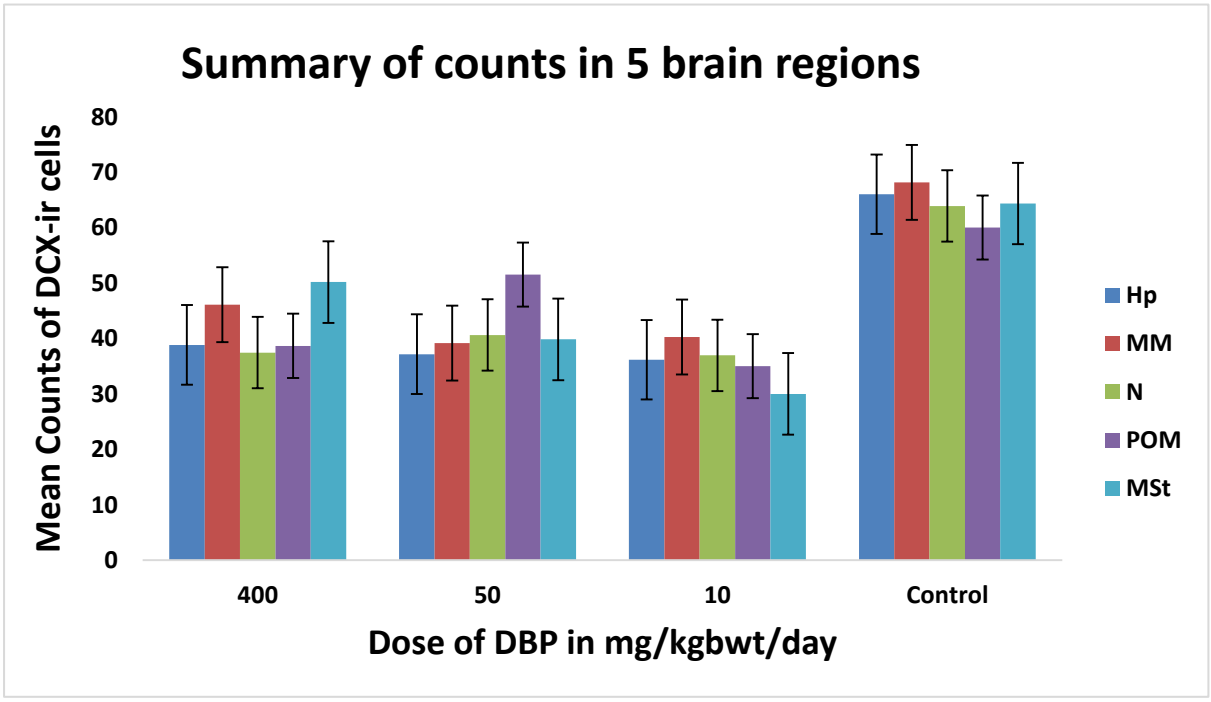


Figure 3.12: Figure A: Graph illustrating the normal distribution of DCX-ir cell counts. Figure B: illustrates DCX-ir cell counts across the 5 brain regions, and across all DBP dosage groups represented as bar graph. Counts were significantly different between the control and treated groups.

Further data analysis examined whether there were any differences in counts between the three rostro-caudal sections (R1, R2 and R3). Results from one way ANOVA showed that there were no significant differences in counts between rostro-caudal sections ($F = 0.28$ $p= 0.75$). Secondly, differences in counts between the treated groups when compared to the control were examined. Linear regression showed evidence that counts were significantly different between the treated groups relative to the control group ($p < 0.0001$).

Thirdly individual differences in counts between the groups themselves in each region compared to the control were examined using linear regression. Results showed evidence that counts were significantly different between individual treated groups compared to the control in all the regions (Hp: $p = 0.0075$, IMM: $p = 0.0001$, N: $p = 0.0001$, POM: $p = 0.0067$ and MSt: $p = 0.0004$). Lastly, data were analysed for any differences between the regions themselves without a comparison to the control. To address this, linear regression was again utilised. There was no evidence to show any significant differences between the regions ($p = 0.886$).

The results from the multiple linear regression, as summarized in table 3.3 show statistical significance ($p < 0.001$) that only the dose was an important predictor of count while region and rostro-caudal position were not. The counts decreased with decrease in the dose. Despite statistical insignificance, higher counts were likely to be in the regions of the IMM and POM. N was lowest likely count compared to Hp.

Table 3.2: Summary of a comparison of counts across rostrocaudal levels, different brain regions and DBP dosage groups in adult male Japanese quail (Coturnix coturnix japonica) brains.

		Coefficient	95% Confidence Interval	P-value
Dose (mg/kgbwt)	400	-23.27	[-28.62: -17.92]	
	50	-23.27	[-29.06: -18.36]	0.001*
	10	-28.23	[-33.58: -22.88]	
	Control	0 (Reference)		
Region	Hp	0 (Reference)		
	MM	4.12	[-1.60: 9.84]	
	N	0.51	[-4.88: 5.9]	0.963
	POM	2.86	[-3.74: 9.46]	
	MSt	2	[-4.6: 8.6]	
Section	R1	0 (Reference)		
	R2	-0.46	[-4.51: 3.58]	0.232
	R3	-3.02	[-7.06: 1.02]	

Mean variables are significant if p<0.05

3.3.4 Numbers of PCNA-ir cells

PCNA-ir cells were quantified in the ventricles and POM. Representative photomicrographs showing the quantification areas and examples of PCNA-ir cells are shown in figure 3.13 and figure 3.14. Table 3.4 shows a summary of the counts. Counts from the POM control group had the highest number (60.00±8.19) while within the ventricles the 400mg group showed the lowest counts (20.5 ±5.79).

Figure 3.16 shows a summary statistic for the response variable and independent variables. Distribution of the counts showed evidence of normal distribution. The bar graph represents counts in the POM and ventricles (Figure 3.13).

Differences in counts of the three rostro-caudal sections (R1, R2 and R3) were initially examined using one way ANOVA for both the POM and ventricles. There was no evidence of significant differences in counts in the three rostro-caudal sections of the POM (F =0.18 p=0.836) and the ventricles (F=0.08 p=0.9218). Linear regression was used to compare differences in counts between the treated

groups and the control. There were insignificant differences between the treated groups and the control for the POM (p=0.0067) while there were significant differences between the control and ventricles (p=0.0001). Results are summarised in table 3.3.

Table 3.3: A Summary of PCNA-ir cell numbers in the medial pre-optic nucleus (POM) and lateral ventricles (VL) in the adult male Japanese quail brain. Results are expressed as the mean± standard deviation (SD) of the mean.

Dose (mg)	**Ventricles**	**POM**
control	58.33(11.85)	60.00(8.19)
10	37.67(3.27)	35.00(6.96)
50	22.5(5.89)	51.50(12.55)
400	20.5(5.79)	38.67(10.75)
	p=0.0001	p=0.0067

Mean variables are significant if p<0.05

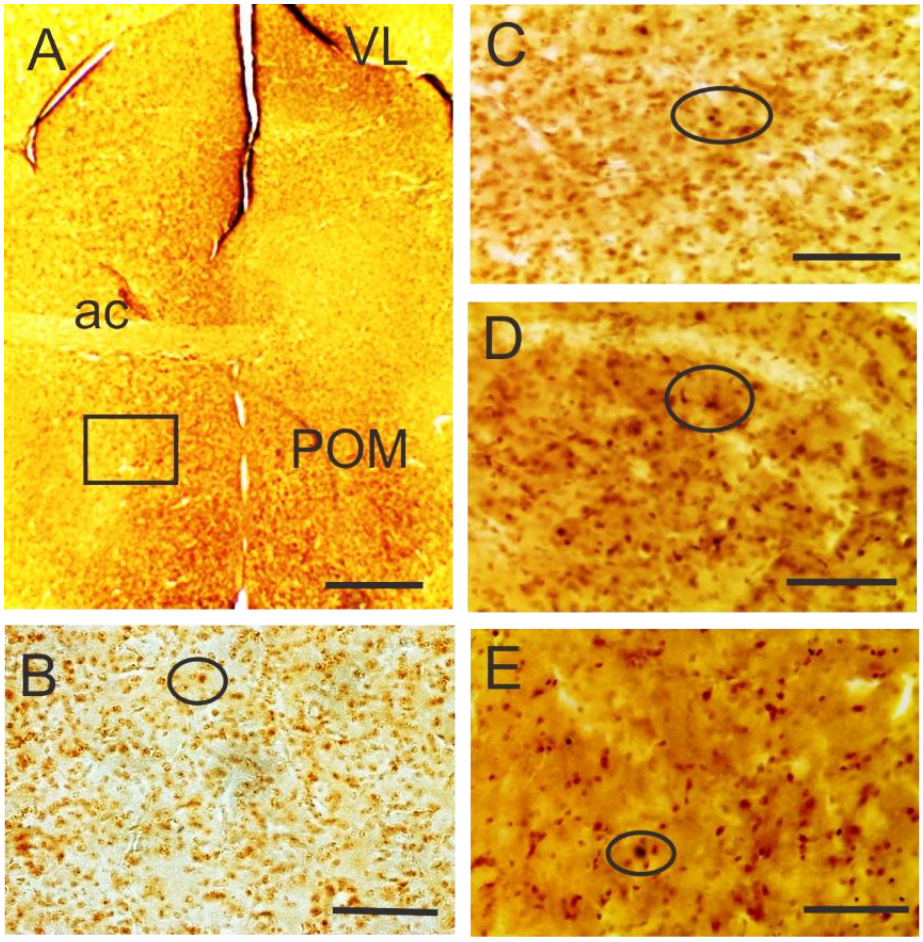


Figure 3.13: Light microscopy photomicrographs of the adult male Japanese quail (Coturnix coturnix japonica) brains following a 30-day pre-pubertal exposure to DBP.

The box in **A** indicates the quantification field within the medial pre-optic nucleus (POM) below the anterior commissure (ac). Photomicrographs **B**, **C**, **D** and **E** represent the DBP control, low (10mg), medium, (50mg) and high (400mg) dose groups respectively. Ellipses within **B**, **C**, **D** and **E** indicate some PCNA positive cells, where the cell density is lower at the high dose compared to the control (B), low (C) and medium (D) doses. VL, ventricles. Scale bar: **A** = X100, **B**, **C**, **D** and **E** = X400. PCNA stain.

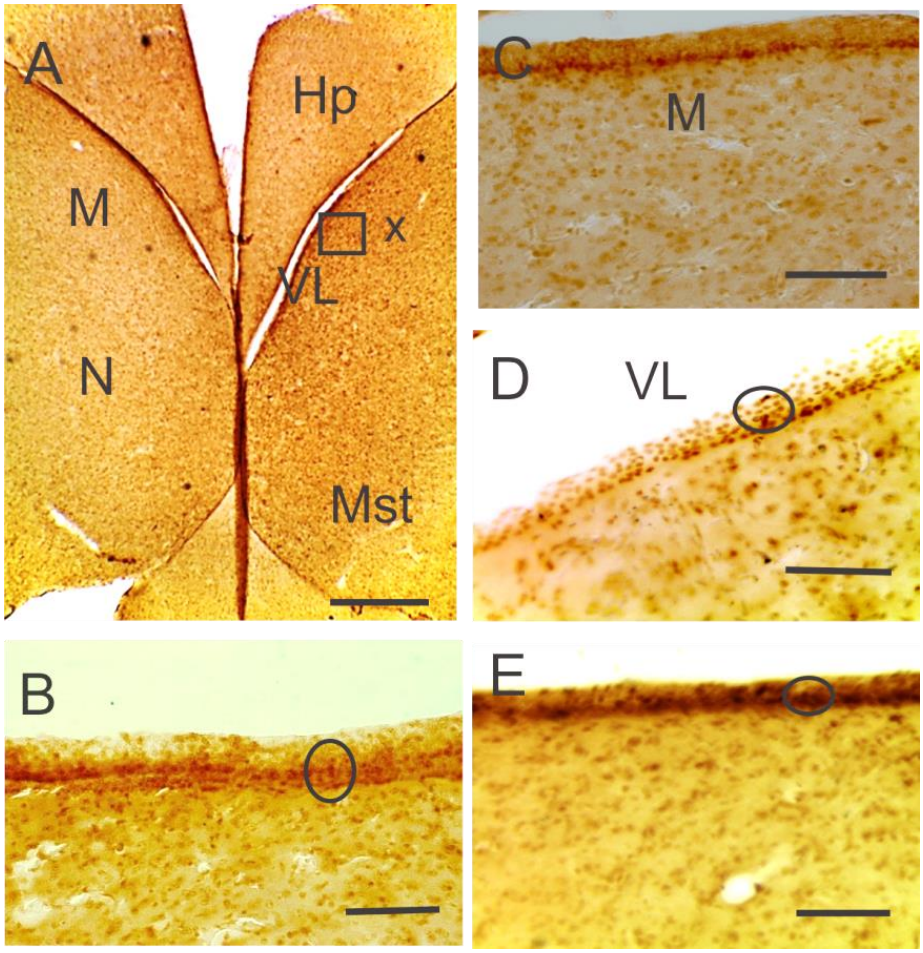
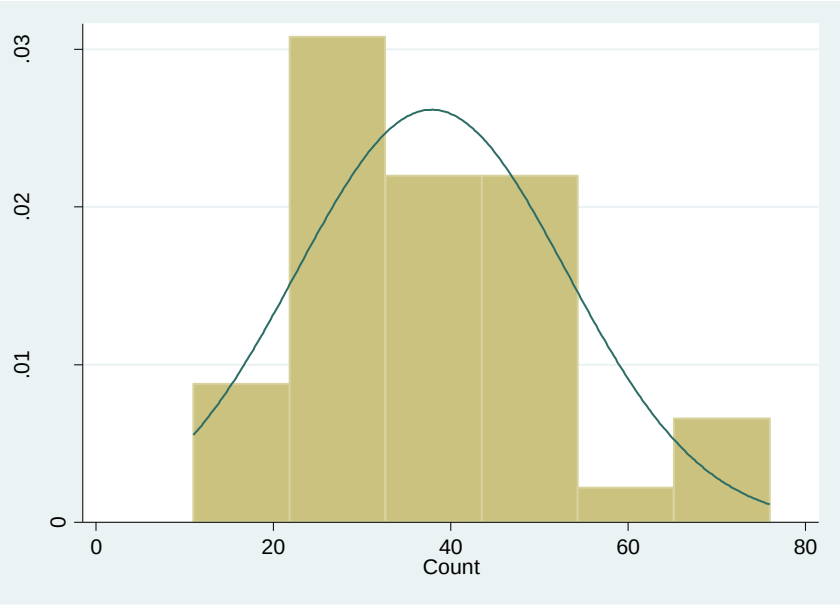


Figure 3.14: Light microscopy photomicrographs of the adult male Japanese quail (Coturnix coturnix japonica) brains following a 30-day pre-pubertal exposure to DBP.

The square marked X in **A** indicates the quantification area of the ventricles overlying the mesopallium (M). Photomicrographs **B**, **C**, **D** and **E** represent the DBP control, low (10mg), medium, (50mg) and high (400mg) dose respectively. The ellipses within **B**, **C**, **D** and **E** represent layers of ependymal and neurogenic cells lining the ventricles. The thickness of this layer appears to decline with increases in dosage. Hp, hippocampus; N, nidopalluim, MSt, medial striatum. Scale bar: **A** = X100, **B**, **C**, **D** and **E** = X400. PCNA stain.

A



B

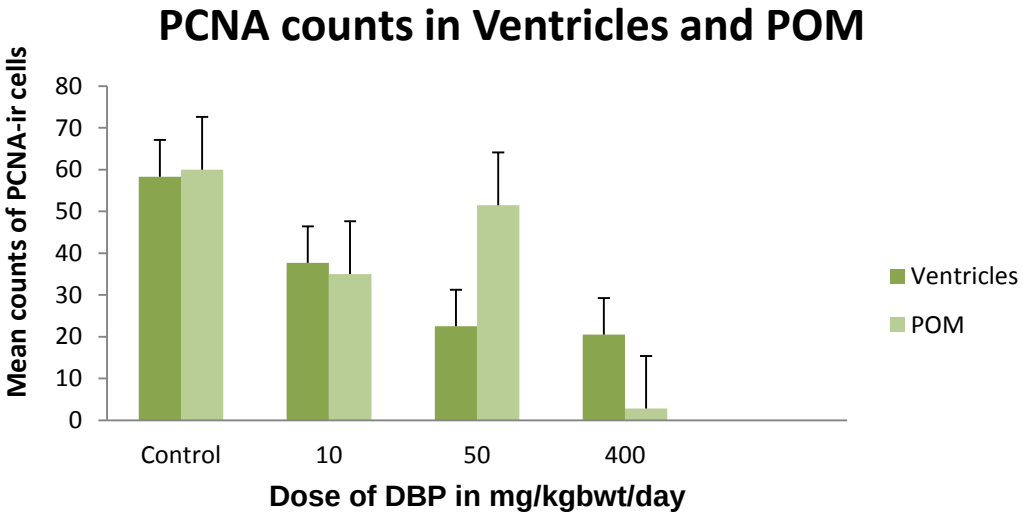


Figure 3.15: Figure A: graph illustrating the normal distribution of PCNA-ir cell counts. Figure B illustrates PCNA-ir cell counts across all DBP dosage groups represented as bar graph. Counts were significantly different between the treated groups relative to the control group ($p < 0.0001$) in the ventricles, while mean counts were insignificant ($p = 0.0067$) between the treated groups and control in the POM

4 CHAPTER 4: DISCUSSION

4.1 General Considerations

An analysis of the effects of DBP was done on adult male quail brains following exposure to various doses of DBP (10 mg/kg, 50 mg/kg, 400 mg/kg), a recognised environmental contaminant with oestrogenic and anti-androgenic properties, which was found to be neurotoxic in neonatal and immature rat offspring (Li et al., 2014). The doses were considered as low (10 mg), medium (50 mg), and high (400 mg) (Bello et al., 2014; Kim and Jeon, 2015).

Previous studies have documented that susceptibility to EDCs is more potent during specific developmental periods especially the foetal, perinatal, peri-pubertal, and early developmental periods (Damstra et al., 2002), hence this study focused on the peri-pubertal/adult period. However the definition of a 'pubertal or pre-pubertal and peri-pubertal quail' remains controversial since Bardet et al. (2012) describe four week old quail as pre-pubertal, seven week old as pubertal and eight week old as a young adult. Ling et al. (1997) regards 12 week old quail as juvenile, Halldin et al. (2006) regards five week old quail as pre-pubertal while Bello et al. (2014) regards 14 week old quail as adults. In this study, 14 week old quail were treated as adults.

Immuno-histochemical methods were chosen to analyse the distribution of DXC-ir cells and PCNA-ir cells using the relevant markers. We confirm the presence of DCX-ir and PCNA-ir cells in the telencephalon of adult quail brains with statistically significant differences between the control and treated groups ($p<0.0001$).

Anti DCX neuroanatomical distribution was found to follow a similar pattern found in studies done on other avian species like parakeets, canaries, zebra finches and

chickens (Bardet et al., 2012; Melleu et al., 2013). DCX is part of the MAP family expressed in the cellular body and processes of post mitotic, migrating and differentiating neurons of developing and adult rat brains and has been proposed as a viable marker to study neurogenesis in vertebrate species. DCX controls polymerisation and stabilisation of the leading process during migration. The laminar arrangement of the cerebral cortex is disrupted when the X-linked DCX gene is interfered with in humans (Boseret el al., 2007; Balthazart et al., 2010; Vellema et al., 2014). Nissl stained sections did not show any cytoarchitectural disruption.

DCX is an extensively researched marker that has been used in human, rodent, and avian species to identify neuronal progenitors, immature neurons, migrating neurons, differentiating neurons and those neurons undergoing dendritic arborisation, axonal outgrowth and synaptogenesis (Boseret et al., 2007; Melleu et al., 2013). The anti DCX antibody used in this study recognises DCX as illustrated by the morphology of DCX-ir cells, which compares with other previous studies, namely small round, fusiform uni/bipolar, and larger multipolar cells (Mezey et al., 2012; Melleu et al., 2013). We quantified those cells with stained cytoplasm, stained processes and no nuclear staining. Most DCX-ir cells appeared to be mainly neuronal progenitors (small round), a few fusiform unipolar or bipolar and occasional large multipolar cells, which were found in different areas of the telencephalon of the control group. Cellular processes were absent in the treated groups, raising the possibility that DBP may affect arborisation in proliferating, migratory and resident cells that have reached their destination. Vellema et al. (2014) caution against the reliability of DCX as a marker to quantify adult neurogenesis because DCX was found in brain regions that do not incorporate young neurons, secondly it was found in mature neurons of

up to one year of age, where these neurons were possibly undergoing dendritic arborisation. He also found DCX to be species specific and brain region specific, following studies on canaries and knockout mice. However, Balthazart and Ball (2014) confirmed the reliability of DCX as a neuronal marker in avian species. Our study focused on a single species, single sex, a specific developmental stage and specific brain regions across all treatment groups and control in alignment with previous studies.

PCNA is a proliferating cell nuclear antigen used as a cell cycling marker which has been used in a variety of avian species (parakeets, quail, doves, zebra finch) and mammals to stain proliferating cells and undertake cellular proliferation studies (Charvet and Striedtar, 2008; Bardet et al., 2012). The anti PCNA staining pattern was predominant along the lateral ventricle, ventricles of the OB and third ventricle which compares with previous studies done on avian species (Charvet and Striedtar 2008; Bardet et al., 2012). On inspection of Nissl stained sections, there was no disruption on the layout of various areas of the telencephalon. The treated and control groups displayed a typical avian brain cytoarchitectural arrangement.

The ventricles were selected because they are neurogenic hotspots with extensive cellular proliferation while pallial areas known to incorporate young neurons like the mesopallium, nidopallium, medial striatum and hippocampus were selected (Barnea et al., 2011; Balthazart and Ball, 2014; Melleu et al., 2016). The POM, a sexually dimorphic nucleus, was selected because it is influenced by testosterone and oestrogen levels (Balthazart et al., 2010). Oestrogen is necessary for neuronal survival and testosterone is aromatised into oestrogen in brain tissue (Halldin et al.,

2006; Lee et al., 2007; Handa et al., 2012). With DBP being neurotoxic, apoptotic, oestrogenic and anti-androgenic, it was relevant to quantify cell numbers in these particular brain regions, namely the POM, N, M, Hp and VL. Endogenous oestrogens bind to ERα and ERβ in brain tissue to promote their neuroprotective effects and DBP also binds to both receptors (Takuechi et al., 2005). In an EDC activity test, DBP was found to have enhanced oestrogenic effects, and induced cell apoptosis in neonatal and immature rat offspring, whereas its sister compound MEHP, and its metabolite DEHP, was found to promote neurocyte differentiation, but suppressed neurocyte proliferation (Li et al., 2013; Qu et al., 2013; Chen et al., 2014; Li et al., 2014). In this study, there were statistically significant differences between the control and all treated groups ($p<0.0001$), which suggests that DBP may have induced cell apoptosis and possibly reduced cell proliferation. The general trend was that counts decreased with increases in dosage, even though most EDCs do not conform to the classical toxicology dose response relationship (Bergman et al., 2012), where effects increase with dosage.

4.2 Nidopallium, Intermediate mesopalluim, Hippocampus, Medial Striatum.

While neuronal birth occurs at the ventricular zone, neuronal survival is determined during migration from the ventricular zone to various brain areas and neuronal recruitment occurs at the final destination which takes approximately three weeks to travel from birth site to recruitment site; it is also assumed neuronal birth is continuous at the ventricular zone (Barnea et al., 2009; Balthazart et al., 2012).

Neuronal recruitment is widespread in the avian telencephalon and areas that were found to recruit young neurons include the mesopallium, nidopallium, hippocampus, medial striatum, which are the focus of the current study. DCX-mRNA and DCX

protein has been identified in avian brains, including Japanese quail, and it is expressed in telencephalic areas that incorporate young neurons (Nikolopolou et al., 2006; Melleu et al.,2013).

A study on zebra finches found moderate DCX expression in the mesopallium, medial striatum and hippocampus while there was a reduced expression in the nidopallium and a more pronounced expression along the ventricles (Kim et al., 2006), whereas a study on canaries, DCX was densely expressed in the mesopallium, lateral and medial nidopallium, medial striatum, walls of the lateral ventricles and hippocampus (Boseret et al., 2007). Vellema et al. (2013) describes high levels of expression in the mesopallium, caudal nidopallium, medial striatum and lateral ventricles with an intermediate expression in the nidopallium and hippocampus in canaries. This neuroanatomical pattern of DCX expression was similar to our findings where there was a strong expression along the walls of lateral ventricles. Higher counts were likely to be in the IMM and the lowest counts in the nidopallium. However, statistical analysis to investigate differences in counts between the various doses and regions showed strong evidence that the counts were significantly different between the doses in each region compared to the control (Hp: $p = 0.0075$, IMM: $p = 0.0001$, N: $p = 0.0001$, POM: $p = 0.0067$ and MSt: $p = 0.0004$). This suggests that DBP treatment affected DCX expression in all brain areas under study, but there were no significant differences between the regions themselves ($p=0.886$). This suggests that exposure of the male Japanese pre-pubertal quail to DBP affected the number of DCX positive cells irrespective of whether the dose was low, medium or high. A study on rats by Kim and Jeon (2015)

also found a reduced expression of DCX in the hippocampus following exposure to DBP in the medium dose (50mg).

Boseret et al. (2007) and Melleu et al. (2013) describe round unipolar/bipolar, fusiform unipolar/bipolar, large multipolar DCX-ir cells within the canary and pigeon telencephalon localised in different areas of the telencephalon, with the nidopallium having more fusiform cells. Boseret et al. (2007) and Melleu et al. (2013) found the nidopallium to be populated by large polygonal multipolar cells with fewer numbers of small unipolar/bipolar and fusiform unipolar/bipolar cells. In this study, we identified a few fusiform uni/bipolar and multipolar cells in the mesopallium, nidopallium and medial striatum with pale staining nuclei and stained cytoplasm and processes. The control had identifiable cell processes while the treated groups had very few cells with identifiable cell processes, suggesting that DBP might affect cellular arborisation and process formation.

The intermediate mesopallium (IMM) is associated with memory formation, memory retention, and the testosterone dependent appetitive behaviour in male quail (Nikolopolou et al., 2006). In chickens, the IMM is responsible for object recognition, filial imprinting and passive avoidance learning, whereby all these characteristics depend on neurogenesis within the IMM (Mezey et al., 2012). Quail and chicken are both non-oscine birds. This suggests that the IMM requires extensive cellular proliferation in non-oscine birds, which is in agreement with our study which found the highest counts within the IMM (68.17±6.46). The nidopallium, medial striatum and hippocampus recruit young neurons where the nidopallium processes somatosensory inputs while the hippocampus exhibits extensive

neurogenesis to augment memory in migratory, food storing and homing birds (Boseret et al., 2008; Balthazart and Ball, 2014). All these areas under study require enhanced cellular proliferation, neuronal migration, neuronal survival and ultimately recruitment and integration into existing circuits.

Neuronal recruitment and survival is under the influence of oestrogen, while testosterone was found to increase neuronal recruitment and survival in the canary HVC (Balthazart et al., 2008). Endogenous oestrogens are synthesised by gonads or via the aromatisation of testosterone in the brain (Balthazart et al.,1993). Testosterone aromatisation into oestrogen modulates the expression of DCX in brain tissue, secondly, DCX-ir neurons in the rat dentate gyrus were found to express oestrogen receptors, confirming a link between the regulation of DCX by oestrogen (Balthazart et al., 2008). Oestrogen receptor labelled cells were observed in the quail telencephalon (Balthazart et al., 1993). DBP has enhanced-oestrogenic and anti-androgenic properties, it binds to ERα and ERβ which were found to be widespread in the quail telencephalon (Li et al., 2014; Qu et al., 2013), therefore cell proliferation should have been enhanced, which is not the case in this study. This could be attributed to selective apoptosis, a natural process that selectively gets rid of newborn and immature neurons in order to control neuronal densities in specific brain regions (Nikolopolou et al., 2006). However, there were no statistically significant differences in counts between the regions ($p=0.886$) which implies there is a possibility that DBP induced non-selective apoptosis across all brain regions. Secondly, according to Barnea et al. (2009), neurotropic factors that promote neuronal survival also promote apoptosis of older neurons. DCX was observed in adult neurons and birth dated neurons of up to one year (Vellema et al., 2014). DBP

could have induced apoptosis of older neurons. Indeed, DBP was found to induce apoptosis and reduce expression of DCX in immature rat offspring (Chen et al., 2011; Kim and Jeon, 2015). DBP was also found to be highly toxic, induced cell apoptosis in neonatal and immature rat offspring while its sister compound, MEHP, was found to suppress neurocyte proliferation in vitro (Chen et al., 2011; Chen et al., 2014).

Testosterone was shown to enhance neuronal differentiation and survival in rats; in songbirds, it enhanced neuronal recruitment and survival in the HVC; in quail, testosterone enhanced cellular proliferation (Nikolopolou et al., 2006; Balthazart et al., 2008; Qu et al., 2013). The control of cell apoptosis and survival by testosterone and oestrogen, namely neuronal turnover, is influenced by brain region specificity and brain function (Nikolopolou et al., 2006). Because of its anti-androgenic properties, DBP could have reduced levels of available testosterone to be aromatised into oestrogen whereby oestrogen promotes neuronal survival. On the other hand, this contradicts findings by Bello et al. (2014) which found that testosterone levels were not affected by the administration of similar doses of DBP in in pre-pubertal quail.

4.3 Medial pre-optic nucleus (POM)

Japanese quail brains are differentiated into male and female during embryonic development before day 12 of incubation (Bardet et al., 2012).

Male sexual behaviour like strutting, crowing, appetitive and consummatory behaviour are all under the influence of testosterone and oestrogen which is enhanced during the peri-pubertal period. The significance of these two hormones is demonstrated by castration which abolishes these traits in quail, but is restored by

exogenous testosterone and synthetic oestrogen (Halldin et al., 2006; Balthazart et al., 2010). Aromatisation of testosterone to oestrogen occurs within the POM where the POM mediates a wide range of behaviours. Oestrogen, not testosterone, is responsible for male sexual behaviour (Halldin et al 2006). In this study, we sought to quantify DCX-ir cells and PCNA-ir cell in the POM because the POM is under the influence of testosterone which becomes significant in the peri-pubertal period. Counts from the POM control group had the highest number (60.00±8.19) while the low dose DBP group had the lowest count (35.00±6.96). Puberty is regarded as the second critical stage for brain organisation and behaviour where both are under the influence of sex steroids (Balthazart and Ball. 2012). ERα and ERβ were highly expressed within the POM of adult male and female quail (Haldin et al., 2006). DBP has enhanced oestrogenic effects and has an affinity for both oestrogen receptor isoforms (Haldin et al., 2006; Chen et al., 2014), therefore, DBP should affect the plasticity of the POM. DBP was found to be anti-androgenic, it induced cell apoptosis and up regulated the expression of aromatase in brains of immature rat offspring, but not in mature offspring (Li et al., 2014). DBP should reduce the levels of testosterone which in turn, should affect aromatase and reduce oestrogen levels.

However, in a concurrent study by Bello et al. (2014) on the same animals, the various doses of DBP did not elicit any significant effects on serum testosterone levels. Testosterone enhances neuronal proliferation, differentiation and survival, after its conversion to oestrogen by aromatase (Li et al., 2014). The anti-androgenic properties of DBP should have affected testosterone levels which means other factors must have contributed to the significant result in cell counts ($p=0.0067$) within the POM between the control and treated groups. Nikolopolou et al. (2006) reported that neuronal turnover is influenced by brain region specificity and brain function,

while Li et al. (2014) found that DBP reduced cell proliferation and induced cell apoptosis in the brains of immature rat offspring, but not adults. DBP could have reduced cell proliferation and possibly induced cell apoptosis within the POM of the adult male Japanese quail, which led to the significant result (p=0.0067) between the control and treated groups. An apoptosis cellular marker should be employed in further studies.

4.4 Lateral ventricles (VL)

Neurogenesis is a multistep process that comprises the birth of new cells from neuronal progenitor stem cells, differentiation into specific phenotypes, migration into specific brain areas, recruitment and integration into existing neuronal circuits, while simultaneously inducing the apoptosis of older neurons (Barnea and Pravosudov, 2011). A study by Alvarez-Buylla et al. (1994) on songbirds identified three cell types within the ventricular zone, namely type B cells (primary precursors or radial glial cells) that undergo mitosis and give rise to type A cells that assume the morphology of migrating neurons, and type E cells (ependymal cells) found along the ventricular surface, together with B cells. The type B or radial glial cells facilitate the migration of type A cells away from the ventricles towards multiple telencephalic areas that incorporate young neurons (Barnea and Pravosudov, 2011). Therefore, cells along the ventricles will express the neuronal marker DCX, and cell cycling marker, PCNA, which were used in this study. Indeed Balthazart et al. (2008) identified DCX-ir cells with a leading and trailing process typical of migrating cells with an orientation parallel to the ventricles whereas Charvet and Striedtar (2008) used PCNA on bobwhite quail to study cell cycling rates within the ventricles.

Cellular proliferation along the ventricular zone continues into adulthood, but declines with age as described by Mezey et al. (2012) on domestic chickens. Two

‘hot spots’ were identified within the ventricles, a ventral hotspot adjacent to the striatum and a dorsal hotspot adjacent to the mesopallium and ventral hyperpallium (Alvarez-Buylla et al., 1994; Reiner et al., 2004). The quantification field in this study was located on the ventriclular zone hot spot adjacent to the mesopallium.

Studies on rats showed testosterone to influence neuronal differentiation and survival embryonically whereas in songbirds, it influenced recruitment into the HVC, but did not influence cellular proliferation in its ventricular zone (Balthazart et al., 2008). In Japanese quail, testosterone was shown to promote cellular proliferation in the male mesopallial ventricular zone, resulting in a higher number of Bird-U-ir cells being incorporated into the male IMM than females (Nikolakopoulou et al., 2006). Oestrogen was found to increase cellular proliferation in the dentate gyrus of female adult rats compared to males. Secondly, oestradiol (E_2) was found to promote rat hippocampal neural progenitor cell proliferation and was found to be higher in pro-oestrus females and breast feeding male pups receiving oestradiol from breast milk (Handa et al., 2012). On the other hand, Barnea and Pravosudov (2011) argue that while testosterone regulates BDNF which increases neuronal recruitment and survival, its effects were found to be short lived (14 -20 days post cell birth) within the HVC. Secondly, oestrogen influences initial neuronal migration, differentiation and survival in avian brains, however both oestrogen and testosterone have no influence on cell division within the ventricles. Cellular decline was observed in the treated groups compared to the DBP control (58.33±11.85), DBP low dose (37.67±3.27), DBP medium dose (22.5±5.89) and DBP high dose (20.5±5.79). Significant statistical differences ($p=0.0001$) were observed between the control and treated groups, possibly due to the direct toxic and apoptotic effects of DBP.

Its anti-androgenic and enhanced oestrogenic can be discounted because oestrogen and testosterone have no effects on ventricular cellular proliferation (Barnea and Pravosudov, 2011).

5 CHAPTER 5: CONCLUSION AND FURTHER STUDIES

5.1 Conclusion

The oestrogenic effects of DBP and resultant neurotropic effects derived from its oestrogenic effects should have enhanced neuronal survival, thus maintaining higher cellular numbers. However, this trend was not observed in this study, instead cell numbers of treated groups were significantly lower when compared to the control. Our test chemical and endocrine disruptor, DBP, with its anti-androgenic and enhanced oestrogenic properties, had a statistically significant effect on cellular proliferation along the ventricles when compared to the control, possibly due to its direct toxicity and apoptotic effects. Oestrogen and testosterone were found to have no effect on cell proliferation within the ventricles. The ventricles are regarded as the birth place of neuronal progenitors that differentiate into young, migrating, and adult neurons. These neurons will eventually be integrated into existing neuronal circuits within the telencephalic areas. In this study, DBP was found to negatively affect cell proliferation within the ventricles, which means the cell pool of migrating neurons will be diminished, leading to a reduction in availability of neurons reaching telencephalic areas that recruit young neurons. This explains the statistically significant differences between the control group and experimental groups, as well as the statistically insignificant differences between the quantified telencephalic brain areas. Of particular interest is the IMM which is associated with memory formation and memory retention in male quail. Although the highest counts were found within the IMM, the statistical differences were insignificant. On the other hand, the ventricles give rise to three cell types, primary precursors, neuronal precursor and ependymal cells. All these cells stain positive for the cell cycling marker PCNA used in this study. Therefore, it is not possible to say which cell line was affected by DBP. Assuming the neuronal precursor cell line was not affected, the statistically

significant differences between the control and experimental or treated groups could be attributed to the direct neurotoxic and apoptotic effects of DBP. Cellular proliferation for the cell cycling marker PCNA within the POM was found to be also significant. Previous studies have observed a surge in PCNA-ir within the POM, suggesting an active cell division during the peri-pubertal period, possibly under the influence of testosterone. This was attributed to slow cycling progenitors found within the POM. Any DBP induced cell toxicity and apoptosis could have been offset by this peri-pubertal surge cellular proliferation, but this was not the case. Exposure to DBP appears to interfere with dendritic arborisation where cell processes were observed in the control group, but not in the treated groups. Studies combining cellular apoptosis, glial marker quantification, taken together with the DCX-ir and PCNA-ir cellular quantification would have been beneficial to elucidate whether cellular declines are due to the natural process of cellular apoptosis or DBP induced cellular toxicity, apoptosis and subsequent endocrine disruption.

Drawing our conclusion or attributing observed results solely on the apoptotic, toxicity or steroidal effects of DBP would be misleading as there are other multiple factors at play which should form the basis for future studies. For example, DCX expression declines with age, it is subject to external factors like photoperiods, diurnal rhythms, stress, social environments, plus internal factors like genetics, chemical pathways, receptor specificity, and other non-steroidal hormones.

5.2 Research limitations

- Time and cost constraints in terms of sourcing antibodies and delivery times. Escalating costs of antibodies which did not allow for repeated validation in laboratory techniques.

- Poor condition of some tissues rendering some sections totally unusable with excessive tearing and tissue loss possibly caused by long storage and suboptimal storage conditions
- Leaching of phthalates from laboratory plastic equipment into brain specimens which may influence results.

5.3 Future studies

1. DCX quantification studies could be done in conjunction with other cellular markers for pyknotic cells, glial cells, synapses and dendritic arborisation to assess if exposure to DBP will affect apoptosis and neuronal integration.
2. Rates of migration from the ventricular zone can be investigated to assess if DBP will have an effect on neuronal migration rates.
3. Long term studies at various developmental stages following exposure during embryonic life until adult hood, followed by behavioural studies to assess long term effects of DBP following embryonic exposure.
4. Brain receptor selectivity and specificity for different types of phthalates and endocrine disruptors.

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APPENDIX I: ETHICS WAIVER

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

Registration number: AREC0101210-002

Solomon Mahlangu House
10th Floor, Room 10004
Jorissen Street
Braamfontein, Johannesburg

20 June 2017

To: Whom it may concern,

Re: Waiver from Animal Ethics Screening Committee of the University of the Witwatersrand

This letter is to confirm that Dr Gcwalisile Frances Dlamini (Student No. 693481, School of Anatomical Sciences in the Faculty of Health Sciences, University of the Witwatersrand) does not require full animal ethics clearance for the study titled "The effect of pre-pubertal exposure to di-(n-butyl) phthalate (DBP) on cell proliferation in male Japanese quail (*c. coturnix japonica*) brains".

The study for higher degree purposes (MSc Med Anatomical Sciences) is based on brain tissue samples collected from a study previously approved for Dr Umar Bello titled 'The effect of di(n-butyl) phthalate (DBP) on the reproductive function of the adult male Japanese quail (*Coturnix coturnix japonica*)'. The study was approved by the ethics committee of the University of Pretoria [Ethics Number V058/12, and V058-12 (Amend 2)].
The study is supervised by Professor Amadi Ihunwo (School of Anatomical sciences, University of the Witwatersrand) and Dr Umar Bello.
Should you require any further information, do not hesitate to contact me.
Yours sincerely,

Kennedy Erlwanger
(Chairman: Animal Ethics Screening Committee, University of the Witwatersrand)

Reference: GF Dlamini Waiver 20-06-2017-O

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APPENDIX II: IMMUNOHISTOCHEMISTRY SOLUTIONS

6.1.1 Citrate Buffer pH6

Tri-Sodium Citrate (Di-hydrate)	2.9g
Distilled water	1000ml

Mix to dissolve and adjust pH to 6 with 1M HCl

Add 0.5ml Tween 20

6.1.2 0.1M Phosphate Buffer pH7.4

NaCl	18g
NA_2HPO_4	3.5g
NaH_2PO_4	6.4g
Distilled water	2000ml

6.1.3 Normal Goat serum and Normal Horse serum

This is made in accordance with the number of wells to be used for each section. Each well is approximately 1ml

.

Normal goat serum	3ml/100ml PBS
Normal horse serum	3ml/100ml PBS

6.1.4 Triton

Triton	25ml
Phosphate buffer (0.1M PB)	100ml

6.1.5 Blocking Buffer (BB)

This is made 2hours before use at room temperature under gentle shaking.

Triton 0.25%in PB	stock solution
Normal serum	3%

BSA (granular) 2%

This is made in accordance with the number of millilitres required, which is determined by the number of sections to be processed on that day, and to cover both primary and secondary antibodies.

6.1.6 Primary antibodies

Anti DCX rabbit polyclonal	1: 1000 (BB/ normal rabbit serum)
Anti PCNA mouse monoclonal	1: 500 (BB/ normal horse serum)

6.1.7 Secondary antibodies

Biotinylated goat anti rabbit	1:1000 (BB/normal goat serum)
Biotinlyated horse anti mouse	1: 1000 (BB/ normal horse serum)

6.1.8 ABC

This solution is prepared 30 minutes before use

PB (0.1M)	stock solution
A reactive	40µl/5000µl
B reactive	40µl/5000µl

6.1.9 DAB

Prepare DAB 20 minutes before use

PB (0.1M)	number of millilitres required/determined by number of sections
DAB	0.05g/100ml PB
30% H_2O_2	3.3 µl:1ml (if using 100%peroxide 0.01 µl/100ml)

Pipette 70% of the DAB into the wells. To the remaining 30% add the peroxide while maintaining the dilution of the peroxide on the original volume. Pipette the DAB and peroxide individually into wells while observing for colour change.

6.1.10 0.4M PB

K_2HPO_4	69.67
NaH_2PO_4	48g
Distilled water	1L

6.1.11 4% Paraformaldehyde

To make 1L

Heat 750ml of distilled water to 60^0C

Add 40g of paraformaldehyde and stir until dissolved (10-20min)

Add 250ml of 0.4M PB

Filter and refrigerate (lasts 1week in the fridge and 6 months in the freezer)

N.B paraformaldehyde denatures above 70^0C.

6.1.12 30% Sucrose

To make 1L

Distilled water	750ml
0.4M PB	250ml
Sucrose	300g

Stir until dissolved and refrigerate. Keeps for several months

6.1.12 Chrome alum gelatine solution

Gelatin	1.5g
Chromium potassium sulphate	0.25g
Distilled water	500ml

Heat water to 60^0C and dissolve gelatin completely, then stir in the potassium sulphate. Store the solution in a dust free container at room temperature. Dip racks of clean slides in the warm gelatin solution (40-50^0C). Drain slides onto paper towel and dry in an incubator overnight at 42^oC.

6.1.13 Cresyl Violet Solution

Cresyl Fast Violet acetate	1g
Distilled water	100ml

Allow to mix on a stirrer for 1 week while covered in foil paper. The solution should not be exposed to light.

6.1.14 Endogenous peroxidase inhibitor

100% methanol	50%
PB (0.1M)	50%
H_2O_2	1.66%

1.66% hydrogen peroxide is calculated as PB+methanol divided by 3.12 which gives you the volume of 30% hydrogen peroxide needed.

6.1.15 2X PO_4 Buffer solution (0.244M)

Distilled water	4L
Sodium Hydroxide	30.8g
Sodium dihydrogen orthophosphate anyhydrous (NaH_2PO_4)	117.12g

6.1.16 Antifreeze

Distilled water	300ml
Glycerol	300ml
Ethylene glycol	300ml
2XPO_4 Buffer	100ml

The solution can be kept in the freezer for several months and allows storage of brain tissue for approximately 20yrs at $^{-}20^{0}$C.

APPENDIX III: PROTOCOL FOR IMMUNOHISTOCHEMISTRY

Brains were removed from antifreeze and stored in 30% sucrose overnight prior to sectioning.

Day 1

1. Brain sections washed in 0.1M PB 3x for 10 minutes under gentle shaking
2. Endogenous peroxidase inhibition for 30minutes under gentle shaking
3. Three washes in 0.1M PB at room temperature under gentle shaking
4. Pre- incubation in blocking buffer for 2 hours under gentle shaking
5. Sections were stored in primary antibody and blocking buffer over night

Day 2

1. Three washes in 0.1M PB at room temperature under gentle shaking
2. Sections are stored in ABC complex for 1hour under gentle shaking
3. Three washes in PB for 10minutes each
4. Sections are transferred to 70% DAB in each well
5. The remaining 30% DAB is combined with hydrogen peroxide
6. The hydrogen peroxide and DAB were pipetted into each well and sections were observed for colour change and washed in PB to arrest the DAB reaction.
7. Wash sections twice in PB for 10 minutes each
8. Stained sections were stored in the fridge until mounting

Day 5

1. Mount sections in 0.5% gelatin coated slides and leave them to dry
2. Dehydrate in a series of alcohols

70%	2hrs
95%	2min
100%	5min
100%	5min
Xylene	5min
Xylene	5min

3. Sections were cover slipped in either DPX or Entellon mounting medium.

APPENDIX IV: NISSL STAINING PROTOCOL

The procedure is relevant for sections that are 25-50um thick.

1. Once the Nissl sections have been mounted and are dry, place sections in a solution of 50% alcohol and 50% chloroform overnight. (1part 100%alcohol: 1 part chloroform)
2. Rehydrate in alcohol

100%	5min
100%	5min
95%	2min
70%	2min
50%	2min

3. Cresyl violet 1min
4. Distilled water 1min
5. Dehydrate in alcohol

50%	2min
70%	this can be variable. Optimal staining checked microscopically
95%	2min
100%	5min
Xylene	5min
Xylene	5min

6. Sections were cover slipped with Entellon or DPX mounting medium.

APPPENDIX V: TURNITIN REPORT

693481:Turn_it_in.docx_2.pdf

by Gcwalisile Dlamini

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

In recent years there has been growing scientific, media and public concern over health threats caused by synthetic chemical pollutants that are pervasive and persistent in our environment. These chemical pollutants or their synthetic analogues, otherwise known as Endocrine-disrupting chemicals (EDCs), have the potential to alter or interfere with normal endocrine function; which is directly linked to hormone biosynthesis, transportation, receptor function; and with the overall effects on physiology, behaviour, development and reproduction in both wildlife and human populations (Colborn et al., 1998; DeRosa et al., 1998; US ATSDR, 2001; Bergman et al., 2013).

Humans and wildlife populations are continuously exposed to EDCs or a cocktail of these compounds, which are constantly discharged into the atmosphere and natural aquatic bodies, including soil, air, households, work and even school environmnents (Shea et al., 1980; Kavlocket al., 2002) where exposure is typically via inhalation, ingestion, and dermal contact (Heudorf et al., 2007).

Studies in mammals and lower vertebrates have suggested that EDCs can cause disorders of neuronal differentiation, as well as disrupt the development and function of neurological organs (Li et al., 2013; Li et al., 2014; Varshney et al.,2017). Therefore it is due to safety related issues and arbitrary use of these synthetic chemicals that concerns have been raised by the International Programme on Chemical Safety (IPCS), and these concerns stemmed from the following factors; unexplained increases in endocrine related diseases (where genetic factors had been ruled out); declines in wildlife populations exposed to these chemicals; a data

base of close to 800 known or suspected EDCs which remain untested; non-validated laboratory test methods to identify specific EDCs and their effects; the the susceptibility of organisms to EDCs during fetal development and puberty, the effects of EDCs on brain development which is linked to neuropsychiatric disorders such as autism, attention-deficit/hyperactivity disorder (ADHD), and learning disabilities.

The IPCS established in 1980, is a joint venture of the United Nations Environment Programme (UNEP), and the International Labour Organization (ILO) and the World Health Organization (WHO) where the overall objective is to establish the scientific basis for assessment of risk to human health and the environment from exposure to chemical pollutants. It also recognises that this knowledge gap compromises the protection of both humans and wildlife species from the harmful effects of EDCs (Damstra et al., 2002). Indeed EDCs are now widely recognized as a major environmental issue. Urbanized and industrialized areas have high concentrations of these chemical pollutants (Hodge and Diamond, 2010). The constant production of EDCs, excessive usage, bioaccumulation in water, soil, air and living organisms, coupled with the ability to spread via oceans, air currents and international exchange of goods leads to global exposures (Kajta and Wojtowicz, 2013; Bergman et al., 2013).

Studies on EDCs as isolated chemicals and/or combinations and their mechanistic effects on humans and wildlife populations are still a grey area, where links between specific EDCs/combinations need to be linked to specific species and tissues. Interestingly the indiscriminate and vast use of pesticides such as dichloro-diphenyl-trichloro-ethane (DDT) and [2,2-bis-(p-chlorophenyl)-1,1-dichloroethylene] (DDE) in the 1950s was correlated with egg shell thining in birds and is probably the best

example of reproductive impairment that caused a severe population decline in a number of raptor species in Europe and North America (Koelman et al., 1972; Peakall et al., 1973; Odsjo and Sonnel, 1982; Vos et al., 2000), and which was found to be induced by embryonic exposure to EDCs (Axelsson, 2008). Importantly, studies on the effects of EDCs on brain tissue particularly in avian species are still lacking. The chemical DBP di(n-butyl) phthalate under study, a phthalate ester, classified as an EDC is implicated as a potential neurotoxic agent (Li et al., 2014). It is suggested that exposures tend to be greater during foetal development, the perinatal period, puberty and childhood hence the need to understand exposures during specific developmental periods like the pre-pubertal period (Damstra et al., 2002; Diamanti-Kandarakis et al., 2009; Bergman et al., 2013). Therefore endocrine related effects may be attributed to specific species, developmental stages, specific tissues, specific chemicals and dosage paradigms.

1.2 Background

The field of endocrine disruption has been controversial with both sceptics and endocrinologists being equally vocal concerning the harmful effects of EDCs especially in determining which chemicals are toxic at what doses, which developmental stages are affected, what tissues are affected, what is the period of latency and what individual variations are experienced (Damstra et al., 2002; Diamanti-Kandarakis et al., 2009; Bergman et al., 2013). The controversy is further compounded by difficulties in extrapolating data derived from in vitro studies and wildlife cases to humans and a lack of sensitive test methods and test guidelines (Beronius et al., 2009; Gore and Patisaul, 2010). Before the terminology of EDCs was introduced, neuroendocrinologists had long developed the concept that interference with endogenous hormones during critical developmental periods leads

to permanent behavioural and physiologic changes in adulthood, whereby disruption of pathways regulated by hypothalamic neuro-endocrine circuits disrupts the homeostasis of neuroendocrine processes, therefore indirectly predicting the neuroendocrine disrupting effect of EDCs (Gore and Patisaul, 2010; Bergman et al., 2013). Central neuroendocrine systems are targets for EDCs (Gore, 2010).

1.3 What are Endocrine Disrupting Chemicals?

The term endocrine-disrupting chemicals or EDCs was first introduced in 1991 at the Wingspread conference centre in Racine Wisconsin USA. According to the U.S Environmental Protection Agency (EPA, 2006), endocrine-disruptors are defined as *'chemicals that either mimic or block the effects of hormones at the target receptor/tissue or by directly stimulating or inhibiting production of hormones by the endocrine system'*,whereas the IPCS/WHO, 2002 defines EDCs as *'exogenous substances or their mixtures that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny or (sub) populations'*. These exogenous substances interfere with normal functioning of the endocrine systems of both humans and wildlife; and thereby mimicking the action of endogenous hormones, antagonise the effects of hormones, stimulate or inhibit production, transportation, or degradation of hormones which consequently results in altering hormonal homeostasis, causing an imbalance or disturbance in the regulation of development, growth, reproduction and behaviour (Jensen et al., 1995; Crisp et al., 1998; Jobling and Tyler, 2006; Verma and Rana, 2009).

According to Waye and Tradeau (2011) EDCs should be viewed in a broad context that is not only confined to hormonal systems but viewed as environmental pollutants that can impact brain function in relation to hormonal systems; hence they propose

the introduction of the term 'neuroendocrine disruption' and the introduction of the concept of 'Neuroendocrine Effects of Endocrine Disruptors' (NEED). Therefore neuroendocrine disruptors can be viewed as pollutants in the environment that are capable of acting as agonists/antagonists or modulators of the synthesis and/or metabolism of neuropeptides, neurotransmitters, or neurohormones which subsequently alters diverse physiological, behavioural or hormonal processes eventually affecting an animal's capacity to reproduce, develop, grow and deal with stress and other challenges in its environment (Waye and Tradeau, 2011).

Central neuroendocrine systems control homeostatic processes like growth, reproduction, metabolism, lactation, stress response, and energy balance, where signals are initiated in the hypothalamus and conveyed initially to neural effectors then endocrine effectors that link the brain with peripheral endocrine systems. Neuroendocrine systems integrate hormone based endogenous signals which control processes such as feedback mechanisms, metabolic pathways, pheromones, temperature, photoperiods, population stresses which in turn result in behavioural, physiological and environmental adaptation of all organisms. Therefore, neuroendocrine disruption transcends basic hormonal disruption to affect other neurochemical pathways responsible for physiological and behavioural processes where the end result is an altered endocrine phenotype (Gore, 2010; Gore and Patisaul, 2010; Waye and Tradeau, 2011)

Exposures to EDCs are implicated in a variety of neurological disorders such as autism, ADHD, and learning disabilities, whereby there is alteration of neural transmission and formation of neural networks (Kajta and Wojtowicz, 2013). Therefore, exposure to EDCs during early life can disrupt normal patterns of development and thus alter brain function and disease susceptibility later in life

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